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1 **A corset of adhesions during development establishes**
2 **individual neural stem cell niches and controls adult behaviour**

3

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1 **ABSTRACT**

2 Neural stem cells (NSCs) reside in a defined cellular microenvironment, the niche,
3 which supports the generation and integration of neuronal lineages. The mechanisms
4 building a sophisticated niche structure around NSCs, and their functional relevance
5 for neurogenesis are yet to be understood. In the *Drosophila* larval brain, the cortex
6 glia (CG) encase individual NSC lineages, organizing the stem cell population and
7 newborn neurons into a stereotypic structure. We first found that lineage information
8 is dominant over stem cell fate. We then discovered that, in addition to timing, the
9 balance between multiple adhesion complexes supports the individual encasing of
10 NSC lineages. An intra-lineage adhesion through homophilic Neuroglian interactions
11 provides strong binding between cells of a same lineage, while a weaker interaction
12 through Neurexin-IV exists between CG to NSC lineages. Their loss leads to random,
13 aberrant grouping of several NSC lineages together, and to altered axonal projection
14 of newborn neurons. Further, we link the loss of these two adhesion complexes during
15 development to locomotor hyperactivity in the resulting adults. Altogether, our findings
16 identify a corset of adhesions building a neurogenic niche at the scale of individual
17 stem cell and provide the proof-of-principle that mechanisms supporting niche
18 formation during development define adult behaviour.

19

1 INTRODUCTION

2 Stem cells are multipotent progenitors driving the growth and regeneration of the tissue
3 they reside in through the generation of differentiated cells. Their localisation within the
4 tissue is restricted to carefully arranged cellular microenvironments, or niches, which
5 control their maintenance and activity in response to local and systemic cues [1–3].
6 The niches comprise the stem cell themselves, their newborn progeny and a number
7 of cells of various origins and roles that support stem cell decisions. The diversity of
8 cellular shapes and roles requires a precise spatial organisation to enable proper niche
9 function towards all and every stem cells. Within the central nervous system (CNS) in
10 particular, a highly structured organ dependent on the tight arrangement of cellular
11 connections, the neural stem cell (NSC) niches are anatomically complex
12 microenvironments that must form within such constraint. They comprise multiple cell
13 types such as neurons, various glial cells, vasculature and immune cells [4,5] which
14 are precisely organised with respect to NSCs. While studies have focused on the
15 identification of signaling pathways operating in an established niche and controlling
16 neurogenesis [5–7], how the niche is first spatially built around NSCs, and the
17 importance of its architecture on neurogenesis, from stem cell division to the
18 integration of the newborn neurons, are poorly understood.

19 The *Drosophila* larval CNS offers a genetically powerful model to study interactions
20 within the niche *in vivo*. Similar to mammals, *Drosophila* NSCs, historically called
21 neuroblasts, self-renew to produce neuronal and glial progeny, and their behaviour is
22 controlled by their niche, an exquisitely organised yet less complex structure than its
23 mammalian counterpart.

24 Fly NSCs are born during embryogenesis, during which they cycle to generate primary
25 neurons in a first wave of neurogenesis. They then enter quiescence, a mitotically
26 dormant phase from which they exit to proliferate through the autonomous activation
27 of PI3K/Akt in response to nutrition [8,9]. This post-embryonic, second wave of
28 neurogenesis generates secondary neurons that will make up 90 % of the adult CNS
29 and lasts until the end of the larval stage. NSCs finally differentiate or die by apoptosis
30 after pupariation. Larval NSCs populate the different regions of the CNS, namely the
31 ventral nerve cord (VNC), the central brain (CB) and the optic lobe (OL) (Fig. 1A). They
32 nevertheless display distinct properties, mainly through different modes of division and
33 expression of specific transcription factors (Fig. 1B) [10]. Type I NSCs, the most

34 represented subtype, reside in the CB and VNC, and divide asymmetrically to generate
35 a smaller ganglion mother cell (GMC). GMCs further terminally divide to produce two
36 neurons. Type II NSCs, found exclusively in the CB, represent a smaller population
37 with 8 cells per brain hemisphere [11–13]. Type II NSC self-renewal produces an
38 Intermediate Neural Progenitor (iINP), which undergoes a limited number of
39 asymmetric divisions to produce GMCs that will subsequently divide to give neurons.
40 These different NSCs are embedded within a sophisticated, multi-layered niche made
41 of different cell types (Fig. 1C-D). The blood-brain barrier forms the interface with the
42 systemic environment, and controls NSC reactivation [14] and proliferation [15]. A
43 specific glial subtype, the cortex glia (CG), is in close contact with NSCs and their
44 progeny, and is crucial for NSC proliferation [16–18], resistance to stress [19,20] as
45 well as for the survival of newborn neurons [21]. Remarkably, the CG form a
46 continuous glial network which invades the whole CNS (Fig 1C-D) while building
47 bespoke encasing of individual NSC lineages (comprising NSC, GMC and newborn
48 neurons, as well as INP for Type II NSCs), called CG chambers [21–23]. CG also
49 enwrap in individual encasing primary neurons and, later on, older, mature secondary
50 neurons (Fig. 1D). CG network is progressively built around NSC during larval
51 development in a process which parallels NSC behaviour [21,24,25] (Fig.1E). CG cells,
52 born during embryogenesis, do not form a continuous meshwork nor encase quiescent
53 NSCs at larval hatching. Rather, they initiate growth in response to nutrition, via
54 autonomous activation of the PI3K/Akt pathway, leading to an increase in membrane
55 density yet without NSC encasing. Then, at the time NSCs start dividing, CG enwrap
56 individual NSCs, forming a typical chequerboard structure. They further extend their
57 processes to maintain a fitted chamber structure during neuronal production. The cell
58 bodies of newborn neurons from one NSC lineage are thus initially found clustered
59 together in one CG chamber; as they mature, they will be individually encased by the
60 CG. Newborn, still immature neurons from the same lineage start to extend axonal
61 projections, which are fasciculated together as a bundle and are also encased by the
62 CG (Fig. 1D and Supp. Fig. 1A-A') until they enter the neuropile, a synaptically dense
63 region devoided of cell bodies, where axons connect [26–28]. There, they will be taken
64 care of by other glial cell types [29]. The repeated pattern of individual chamber thus
65 translates both in term of cell bodies and axonal tracts.

66 The reliable formation of such precise chequerboard structure implies that CG
67 integrate proper cellular cues to decide whether to encase specific cells, while
68 navigating between a density of diverse cell types. However, the nature of these cues,
69 and the importance of such stereotypic encasing of NSC lineages on NSC activity and
70 generation of functional neuronal progeny remained to be identified. We first found that
71 CG are able to distinguish between lineages and that lineage information prevails over
72 cell identity. Further, we discovered that lineage information and individual encasing
73 are mediated by the existence of multiple adhesion complexes within the niche. First,
74 the cell adhesion protein Neurexin-IV is expressed and crucial in NSC lineages to
75 maintain their individual encasing, through its interaction with Wrapper, a protein with
76 immunoglobulin domain present in the CG. In parallel, Neuroglian appears to form
77 strong homophilic interactions between cells of the same lineages, keeping them
78 together by providing a stronger adhesion compared to the weaker interaction between
79 CG and NSC lineages. In absence of either Neurexin-IV and Neuroglian, NSC lineages
80 are grouped in a random fashion. The loss of these adhesions is further associated
81 with misprojected axonal bundles. Adherens junctions are also present in NSC
82 lineages, however they appear mostly dispensable for individual encasing. Further, we
83 demonstrated that the loss of Neurexin-IV and Neuroglian adhesions during
84 development is linked to an altered, hyperactive locomotor behaviour in the adult. Our
85 findings unravel a principle of NSC niche organization based on a differential in
86 adhesion and link the adhesive property of the niche during development to adult
87 neurological behaviour.

1 RESULTS

2 Cortex glia distinguish between NSC lineages.

3 Before lineage generation, CG encase only one, and not several NSCs within a
4 membranous chamber, suggesting that CG can sense cell identity to decide which cell
5 to enwrap. To assess the importance of NSC fate in chamber formation, we took
6 advantage of genetic alterations known to dysregulate NSC division and differentiation
7 and lead to the formation of tumour-like, NSC-only, lineages [30]. In particular, *pros*
8 knockdown in Type I lineages converts GMC into NSC-like, Dpn⁺ cells at the expense
9 of neurons [31]. Surprisingly, in these conditions, we found that CG chambers
10 contained not one, but several NSC-like, Dpn⁺ cells (Fig. 1F). Similar results were
11 obtained for other conditions that lead to Type I NSC-only lineages, including GMC
12 dedifferentiation via Dpn overexpression or loss of asymmetric division via aPKC
13 overexpression (Supp. Fig. 1B). We then asked how CG would adapt to the
14 dysregulation of another type of NSCs, the Type II NSC. Since CG chamber formation
15 was precisely described only for type I NSCs [21], we first checked the dynamics of
16 CG morphogenesis around type II and found that they followed similar steps, albeit in
17 a slower fashion (Supp. Fig. 1C). We then knocked down the cell fate determinant *brat*
18 [32,33], which is necessary for preventing iINP dedifferentiation into NSC-like cells.
19 This led to the formation of large tumours of Type II NSCs (Fig 1F). CG were able to
20 adapt to the outgrowth of cells and enwrapped many Type II NSCs within one chamber.
21 These data show that both for Type I and Type II NSCs, stem cell identity is not
22 sufficient to ensure their sorting into individual CG chambers.

23 We then wondered whether tumour NSCs grouped within one chamber originated from
24 the same NSC mother cell or had been encased randomly independently of their
25 lineage of origin. To do so, we used multi-colour clonal analysis to label individual NSC
26 lineages. The Raeppli system [34] allows the stochastic and irreversible labelling of a
27 cell at the time of induction, allowing to mark and track a mother cell and its colour-
28 sharing progeny. Induction before NSC reactivation of a nuclear tagged version of
29 Raeppli (Raeppli-NLS) ensured all lineages could be fully tracked. We first confirmed
30 that cells found within each CG chamber belonged to the same lineage for wild-type
31 Type I and Type II NSC lineages (Fig 1G). We then found that clonal tumour-like growth
32 coming from single dysregulated NSCs were contained within one CG chamber, both
33 for Type I and Type II NSCs (Fig. 1G). Altogether, these results demonstrate that CG

34 are able to distinguish between different NSC lineages, and that keeping a lineage
35 together prevails over encasing individual NSC, thus showing that the lineage
36 information prevails over cell identity.

37 **Individual encasing relies on intrinsic lineage cues**

38 We then assessed the molecular basis of NSC lineage recognition and individual
39 encasing by CG. Chamber completion around NSCs occurs around the time of first
40 division and is driven by NSC reactivation [21,24]. A simple explanation for keeping a
41 lineage together and separated from others would thus be the sequential addition of
42 newborn cells within a compartment already defined from the start, by NSC-derived
43 signals (Fig.1H, Panel I). Timing would thus be the instructive cue. Indeed, previous
44 studies have shown that instructions from reactivated NSCs are paramount to form CG
45 niches [21], although the cues integrated by the glia have not been identified. Within
46 this hypothesis, blocking CG morphogenesis until well after the first NSC division,
47 followed by subsequent release of their growth, would result in aberrant chamber
48 formation and random encasing of neurons from different lineages.

49 To do so, we prevented chamber formation by blocking the activation of the PI3K/Akt
50 pathway (PTEN inhibitor) specifically in the CG [21], using the QF system to control
51 the timing of induction (Fig. 2A). At the same time, NSC lineages were stochastically
52 marked using *Raeppli-NLS*, driven this time by the GAL4/UAS system. CG growth was
53 prevented until NSCs cycled actively and neuronal progeny had been already
54 produced, resulting in CG failing to form correct chambers to separate individual
55 lineages from each other (Supp. Fig. 2A, time T1). We then allowed CG to resume
56 their growth and observed the establishment of a stereotyped chequerboard pattern,
57 with most of the chambers containing single NSC and neurons (Fig. 2B, time T2). This
58 was confirmed by quantifying the chambers containing more than one NSC lineage
59 (Fig. 2C). These results thus show that the information allowing CG to distinguish
60 between lineages is not an exclusive property of the NSC, but is likely inherited by their
61 progeny, and can be sensed by CG later during larval development.

62 One elegant way cells could be recognised by CG as belonging to the same lineage
63 would be to keep them physically together through adhesion mechanisms. We
64 hypothesized two possibilities (Fig. 1H, Panel II).

65 The most complex mechanism would rely on the existence of lineage-specific
66 adhesions, with a code of unique molecular interactions between specific CG cells and

67 all cells of specific lineages (Fig. 1H, Panel II.1). In this context, CG cells would not be
68 interchangeable.

69 The simplest solution would see all NSC lineages using the same adhesion
70 mechanisms to link their cells (Fig. 1H, Panel II.2). The differential adhesion hypothesis
71 proposes that cells with similar adhesive strength cluster together, ultimately sorting
72 cell populations with different adhesions and creating cellular compartments [35].
73 While NSC lineages and CG are not fully sorted in only two compartments, NSC
74 encapsulation could be seen as a local segregation event between whole
75 NSC lineages and the CG. NSC lineages with intra-lineage adhesions (A_L) stronger
76 than their adhesion to the CG (A_{L-CG}) would form a physical barrier for the CG,
77 preventing their intercalation in between cells from the same lineage and thus leading
78 to encapsulation of the whole lineage. In this case, a difference in adhesive properties
79 would be sufficient to segregate CG from NSC lineages, and CG cells would be
80 interchangeable.

81 To discriminate between these two hypotheses, we first assessed the result of
82 preventing some CG cells to cover NSC lineages to a normal extent (Fig. 2D). We
83 used clonal analysis to randomly impair the growth of a few CG cells within the entire
84 population, through a Coin-FLP approach [36]. Blocking PI3K/Akt signaling in a few
85 cells (marked with RFP) through the expression of the inhibitor PTEN resulted in much
86 smaller clones compared to control wild-type clones (Fig. 2E). However, the CG
87 network itself appeared gapless around PTEN clones, revealing that CG were able to
88 compensate for the loss of their neighbours' membrane to restore NSC chambers
89 (Fig. 2E). To confirm that CG cells are completely interchangeable we used the same
90 approach to kill a few CG cells, expressing the pro-apoptotic gene *reaper* [37] to induce
91 apoptosis only once the NSC chambers were already formed (Supp. Fig 2B). Induction
92 of apoptosis led to a near complete loss of RFP-marked clones, only visible through
93 cell remnants. Nevertheless, the whole CG network appeared intact. This shows that
94 any given NSC lineage can recruit other CG cells, and that, in accordance with our
95 previous findings (Fig 2A-C), it is able to do so after NSC reactivation. Similar results
96 demonstrating the ability of CG to replace each other has been previously obtained
97 around neurons [38]. Altogether, our results indicate that NSC lineages can be
98 enwrapped by different CG already taking care of other lineages, and that specific CG-
99 NSC lineage pairings do not occur. This suggests that the same adhesion mechanism

100 might be repeated for each NSC lineage, providing stronger cohesion between cells of
101 the same NSC lineage than between CG and NSC lineages.

102 **Intra-lineage adherens junctions are present but not absolutely required for** 103 **encasing of individual NSC lineages**

104 Previous studies had reported the localization within larval NSC lineages of the
105 *Drosophila* E-cadherin Shotgun (*Shg*), a component of adherens junctions usually
106 present in epithelia [22,39]. We analysed the expression pattern of *Shg* during larval
107 development, using a protein trap fusion (*Shg::GFP*, Fig. 3A). *Shg::GFP* was detected
108 from larval hatching, initially present around and between NSCs (ALH24, dashed
109 yellow circle), and also along CG membranes (white arrowheads). *Shg::GFP* was then
110 no longer detected between NSCs having proceeded through reactivation, yet not
111 individually encased (ALH48, yellow stars). At later larval stages, following neuronal
112 production, *Shg::GFP* showed a remarkable pattern of expression, with a strong
113 enrichment between cells from the same lineage (ALH72). A similar pattern was found
114 for its β -catenin partner Armadillo (Fig. 3B) through antibody staining. As *Shg* form
115 homophilic bonds, this suggests that adherens junctions exist between cells of the
116 same NSC lineage.

117 We then wondered whether such adhesion was a property of differentiating lineages
118 within the CG chamber, or whether it could take place between cells of similar fate and
119 identity. We first looked at NSC-like cells from *pros* and *brat* tumours, which we showed
120 are contained clonally within one CG chamber (Fig. 1G). A strong *Shg* staining was
121 detected between NSC-like cells from the same lineage (Supp. Fig. 3A). It was
122 however not the case when they originated from different lineages, which were
123 separated by CG membrane. This suggests that adherens junctions are not only a
124 property of differentiating lineage, but, importantly, is found between cells in an inverse
125 correlation with the presence of CG membranes. In line with this finding, we observed
126 a strong *Shg* staining between NSCs of the optic lobe, another type of neural
127 progenitors [40] which are contained within one CG chamber (Supp. Fig. 3B).
128 Altogether these results suggest that AJ could be a mean of keeping lineages together,
129 providing differential adhesion that would ensure stronger bond between cells of the
130 same lineage than between lineages and CG, and functioning as a physical barrier to
131 further enwrapping of individual cells by CG membranes.

132 We first asked whether adherens junctions were necessary for keeping cells together
133 within the CG chamber. Previous studies indeed suggested that E-cadherin expression
134 was required in NSC lineages for proper CG structure [22,41]. We first generated NSC
135 lineages mutant for *shg*, by inducing MARCM clones during late embryogenesis (see
136 Methods). To our surprise, Shg-depleted NSC lineages still stayed individually
137 encased within one CG chamber (Supp. Fig. 3C, upper panel). The same result was
138 obtained when clones were induced at a later timepoint, to prevent potential
139 compensation through the upregulation of other adhesion molecules (Supp. Fig. 3C,
140 lower panel). In accordance with these results, we found that RNAi knockdown of *shg*
141 in NSC or neurons did not disrupt overall lineage organization within CG chambers
142 (Fig. 3C), despite successfully removing Shg::GFP signal (Supp. Fig. 3D). To note, we
143 also did not record disruption of CG network when *shg* was knocked down in CG
144 themselves (Supp. Fig 3E), contrary to previous findings [22]. Driving the multicolour
145 clonal marker Raepli-NLS (induced at ALH0) along with *shg* RNAi driven from
146 embryogenesis in the NSC lineage demonstrated the conservation of individual
147 encasing despite efficient E-cadherin loss (Fig. 3D and Supp. Fig. 3F-G). These results
148 argue against the strict requirement of Shg-mediated adhesion for NSC lineage
149 maintenance within one CG chamber [22,41]. All together, these data suggest that
150 adherens junctions, while expressed within the NSC lineage, are not absolutely
151 required for their individual encasing.

152 We then wondered whether intra-lineage AJ could rather be used as a safety
153 mechanism, ensuring robustness in a system in which other strategies would primarily
154 provide intra-lineage cohesion. As the CG chamber encases NSC at the time they
155 produce their first progeny, timing would bring a first level of clustering (Fig. 1H, case
156 I), while AJ would ensure its maintenance in the case the chamber is affected (such
157 as in Fig. 2A-C). To test this hypothesis, we performed a conditional block of CG growth
158 while at the same time constantly driving *shg* RNAi and the multicolour clonal tool
159 Raepli-NLS in NSC lineages (Fig. 3E and Supp. Fig. 3H). The efficiency of *shg*
160 knockdown was assessed through E-cadherin staining (Supp. Fig. 3F). In this case,
161 the progeny born after the re-establishment of CG growth will naturally be encased,
162 while before will depend on the requirement of adherens junctions in absence of proper
163 encasing. Looking at the slightly deeper level in which differentiating progeny reside,
164 we uncovered few restricted, localised defects in the individual encasing of NSC

165 lineages, with several colours detected within the boundaries of one continuous CG
166 membrane (Fig. 3F-G). However, most lineages still appear encased correctly These
167 results indicate that adherens junctions might participate in the robustness of individual
168 NSC lineage encasing when CG are altered, however in a limited fashion.
169 Finally, we wondered whether altering the respective adhesion balance between NSC
170 lineages and CG would shift the site of preferential adhesion, and thus alter the sorting
171 of the different cell types. Ultimately, this would result in randomizing the number of
172 NSC lineage encased within one CG chamber. To do so, we overexpressed *shg* in the
173 CG, with the aim to force the recruitment of lineage-expressed endogenous Shg to
174 adherens junctions artificially set up between CG and NSC lineage cells (Fig. 3H), and
175 as such to flatten the E-cadherin based adhesion difference (with now $A_L = A_{L-CG}$ for
176 Shg). Lineage-expressed endogenous Shg would thus have the choice to generate
177 adhesion of similar strength either with itself, or with CG-provided Shg. Despite the
178 successful expression of *shg* in the CG, the usual pattern of CG chambers was
179 nevertheless maintained in this condition (Fig. 3H), suggesting that in this case A_L still
180 stays superior to A_{L-CG} , and that other adhesions exist to fulfill this role. These data
181 imply that a difference in adhesion using adherens junction is not the main driver for
182 ensuring the individual encasing of NSC lineages.

183 **Occluding junction components are expressed in NSC lineages**

184 These findings prompted us to investigate the potential presence and role of other
185 adhesion complexes which could provide intra-lineage cohesion and differential
186 adhesion to sort NSC lineages from CG.

187 Occluding junctions (tight junctions in vertebrate and septate junctions in *Drosophila*)
188 [42,43] primarily perform a permeability barrier function to paracellular diffusion, mostly
189 described and understood in epithelia or epithelial-like cells. However, they can also
190 provide some adhesion between the cells they link, albeit possibly in a weaker fashion
191 than adherens junctions. *Drosophila* septate junctions are formed by the assembly of
192 cell surface adhesion molecules that can interact in *cis* or *trans*, in an homologous or
193 heterologous fashion, and which are linked to the intracellular milieu by supporting
194 membrane or cytoplasmic molecules [42]. A core, highly conserved tripartite complex
195 of adhesion molecules comprises Neuroglian (Nrg), Contactin (Cont) and Neurexin-IV
196 (Nrx-IV). Nrg, the *Drosophila* homolog of Neurofascin-155, is mostly a homophilic
197 transmembrane protein belonging to the L1-type family and containing several

198 immunoglobulin domains. Cont, homologous to the human Contactin, also contains
199 immunoglobulin domains, is GPI-anchored and only performs heterophilic interactions.
200 Nr_x-IV, homologous to the human Caspr/Paranodin, is transmembrane protein with a
201 large extracellular domain including multiple laminin-G domains and EGF repeats [44]
202 and is able to set up heterophilic interactions. In vertebrates, Caspr/Paranodin and
203 Neurofascin-155 are also partners at the paranodal junction between glia and neuron
204 [42]. Besides adhesion molecules, several cytoplasmic or membrane-associated
205 proteins participate in septate junction formation, such as the FERM-family Coracle
206 (Cora), the MAGUK protein Discs large (Dlg1), and the integral membrane Na K-
207 ATPase pump (ATP α).

208 Interestingly, Nr_x-IV and Nrg also perform nervous system-specific roles outside of the
209 septate junction. Nr_x-IV is required in the embryonic CNS for the wrapping of individual
210 axon fascicles by midline glia [45–47]. Similarly, neuronal expression of Nrg is
211 important for axonal guidance and regulation of dendritic arborization of peripheral
212 neurons by glial [48,49], and epidermal cells [50], as well as for the function and axon
213 branching of specific larval CB neurons [51,52]. For both proteins, their role in NSC
214 lineages during larval neurogenesis is however poorly known.

215 We started by assessing the expression and function of septate junction (SJ)
216 components in the larval CNS, and found that Nr_x-IV, Nrg, Cora, Dlg1 and ATP α are
217 enriched in NSC lineages.

218 First, using a protein trap (*Nr_x-IV::GFP*, Fig. 4A), we found that Nr_x-IV expression was
219 detected from early larval stage in embryonic (primary) neurons, and around NSCs
220 (ALH0), an expression maintained while NSCs proceed through reactivation (ALH24,
221 dashed yellow circle). As NSCs have reactivated and CG grown (ALH48), Nr_x-IV::GFP
222 appears expressed at the interface between NSC and CG (yellow arrow), but not
223 anymore between NSCs (yellow stars). Further, accompanying the production of
224 newborn, secondary neurons (ALH72), Nr_x-IV::GFP is found enriched at the interface
225 of the cells from the same lineage (NSC, GMC and neurons), while maintaining a
226 strong expression at the interface with CG (yellow arrowhead). A protein trap for Nrg
227 (*Nrg::GFP*, Fig. 4B) also revealed a strong enrichment between cells of the same NSC
228 lineages following progeny production (ALH72). At this time, Nrg appears enriched in
229 the axonal bundles leaving from secondary neurons, in accordance with previous
230 findings [53]. Moreover, Nrg::GFP is detected at the interface between lineages and

231 CG (yellow arrow). However, contrary to what we observed with *Nrx-IV::GFP*,
232 *Nrg::GFP* did not appear enriched between NSCs at any time point before their
233 encasing by CG (ALH0-48, yellow stars and dashed yellow circle).

234 Strikingly, the enrichment at the interface between cells from the same NSC lineage
235 was also present for other SJ components, namely *Dlg1*, *ATP α* and *Cora*, with the
236 latter also exhibiting a staining along the CG interface (Supp. Fig. 4A). We were not
237 able to assess *Cont* due to lack of working reagents. Taken together, these data
238 suggest that multiple SJ components are expressed in NSC lineages, localizing
239 between cells of the same lineages, as well as between lineages and the CG.

240 ***Nrx-IV* and *Nrg* are required in NSC lineages for individual encasing by CG**

241 We then asked the importance of such expression in the individual encasing of NSC
242 lineages by CG. We decided to knock down from ALH0 *nrx-IV*, *nrg*, *dlg1*, *cont* and
243 *ATP α* in NSC lineages using specific RNAi lines. Larvae from *dlg1*, *cont* and *ATP α*
244 knockdown died at early larval stages. From *ATP α* knockdown, few larvae still reached
245 late larval stage, displaying restricted irregularities the CG network (Supp. Fig. 4B). In
246 contrast, *nrx-IV* and *nrg* knockdowns, which successfully reduced the expression in
247 NSC lineages of *Nrx-IV::GFP* and *Nrg::GFP*, respectively (Supp. Fig. 4C-D), both
248 resulted in altered encasing of individual NSC lineages.

249 Under *nrx-IV* knockdown, CG pattern first appeared mostly normal when observed at
250 the NSC level, with NSCs seemingly individually separated by CG membranes.
251 However, we observed a striking, unusual pattern at the level of differentiating progeny,
252 with much larger CG chambers harbouring a clear continuous membrane outline with
253 no signal inside (Fig. 4C). A similar result was obtained when *nrx-IV* RNAi was
254 expressed under the control of the neuronal driver *ElaV-GAL4* (Supp Fig. 4E). In
255 contrast, no effect was detected under *nrx-IV* knockdown in CG (Supp. Fig. 4E).
256 Expressing the multicolour clonal marker *Raeppli-NLS* along with *nrx-IV* RNAi in NSC
257 lineages (both induced at ALH0) revealed an extensive loss of individual encasing,
258 with multiple NSC lineages not separated by CG membranes but rather clustered
259 together in large chambers (Fig. 4D-E).

260 Driving *nrg* knockdown from larval hatching (ALH0) led to few larvae of the right
261 genotype, in which CG displayed some restricted defects in individual encasing of NSC
262 lineages. We thought these animals might have survived due to a weak phenotype,
263 and decided to delay the RNAi knockdown, started after one day, and then maintained

264 for 2 to 3 days. In this case, we obtained more surviving larvae which displayed strong
265 alterations of NSC individual encasing by CG. At the stem cell level, NSC were indeed
266 clustered together, seemingly touching each other (Fig. 4F). Going deeper at the level
267 of the maturing progeny also revealed bigger zones devoided of CG membrane (see
268 orthogonal view). Of note, the expressivity of the phenotype was variable (around 30%
269 of the larvae display milder alterations, a population seen and representing the lower
270 points in Fig. 4H). Driving *nrg* RNAi in neurons (ElaV-GAL4) in the same induction
271 conditions also led to defects in the encasing of NSC lineages, albeit in a weaker
272 fashion than with the NSC driver (Supp Fig. 4F). Finally, *nrg* knockdown in CG did not
273 lead to observable CG alteration (Supp. Fig. 4F). Expressing Raeppli -NLS along with
274 *nrg* RNAi in NSC lineages confirmed the loss of individual encasing, with multiple NSC
275 lineages grouped together in large chambers, in a seemingly random fashion (Fig. 4G-
276 H).
277 Altogether, our findings suggest that the expression of Nr_x-IV and Nrg in NSC lineages
278 are both required for their individual encasing by CG.

279 **A glia to NSC lineages adhesion through Nr_x-IV and Wrapper is required for** 280 **individual encasing**

281 The dual role of Nr_x-IV within and outside septate junctions is sustained by the
282 existence of alternative splicing [46]. Nr_x-IV can be produced as a SJ isoform (Nr_x-
283 IV^{exon3}), and a neuronal isoform outside of SJ (Nr_x-IV^{exon4}). Nr_x-IV role within the
284 embryonic CNS is through its recruitment by and binding to its glial partner Wrapper,
285 another member of the immunoglobulin family [45–47]. We thus sought to assess
286 whether the role of Nr_x-IV in NSC lineages encasing by CG was through, or
287 independent of, Wrapper.

288 Previous studies had reported that regulatory sequences in the *wrapper* gene drive in
289 the CG during larval stages [38,54]. Staining with an anti-Wrapper antibody confirmed
290 Wrapper expression in CG, where it localizes in the membrane (Fig. 5A). In addition,
291 a CRIMIC line [55], in which a CRISPR-directed intronic insertion allows *GAL4*
292 expression under control of endogenous *wrapper* promoter, was used to drive
293 fluorescent membrane (*UAS-mCD8::GFP*) and nuclear (*UAS-His::mRFP*) reporters. It
294 produced the stereotypic CG pattern and co-stained with a glial fate marker, Repo
295 (Supp. Fig. 5A). We then found that RNAi knockdown of *wrapper* in the CG reproduced

296 the highly characteristic pattern of large chambers (Fig. 5B) found during Nr_x-IV
297 knockdown in NSC lineages (compare with Fig. 4C). Taken together, our results
298 suggest that Nr_x-IV in NSC lineages partners with Wrapper in the CG, outside of a
299 septate junction function. Moreover, this interaction is essential to produce individual
300 encasing of NSC lineages by CG, implying that in absence of such recognition, CG
301 cannot properly recognize and individually sort NSC lineages.

302 We then wondered how the Nr_x-IV to Wrapper interaction would fit in the hypothesis
303 of a difference in adhesion between A_L and A_{L-CG}. If CG to NSC lineage adhesion is
304 indeed weaker than intra-lineage adhesion (Fig. 1H, panel II.2), shifting Nr_x-IV binding
305 to Wrapper from a glial to a NSC lineage pool (thus providing intra-lineage adhesion
306 through Nr_x-IV and Wrapper in addition to other existing interactions) should not affect
307 the sorting between NSC lineages and CG, since A_L would still be superior to A_{L-CG}.
308 However, if Nr_x-IV to Wrapper interaction is stronger than the sum of intra-lineage
309 adhesions, then forcing it in the lineage would tend to flatten the difference in adhesion
310 ($A_L \approx A_{L-CG}$), and thus lead to random grouping of NSC lineages together. We found that
311 misexpressing *wrapper* in NSC lineages from larval hatching (ALH0), while successful,
312 resulted in very little alteration of CG encasing of individual NSC lineages (Fig. 5C).
313 These data plead in favour of a CG to NSC lineage adhesion through Nr_x-IV and
314 Wrapper being weaker than the sum of intra-lineage adhesions.

315 **Intra-lineage adhesion through Nrg drives individual encasing by CG**

316 Similarly to Nr_x-IV, the dual role of Nrg in and outside of septate junction bores from
317 differential splicing [56]. Nrg indeed comes in two isoforms, with the same extracellular
318 domain but different intracellular parts (Fig. 5D). While the short isoform, Nrg¹⁶⁷,
319 localizes in the SJ of epithelial tissues, the long isoform, Nrg¹⁸⁰, is expressed in
320 neurons of the central and peripheral nervous systems during development [50,57,59].
321 We first determined which isoform is expressed in NSC lineages during larval stage,
322 taking advantage of an antibody (BP104) specifically recognizing the Nrg¹⁸⁰ isoform
323 [56]. Staining of *Nrg::GFP* CNS (ALH72) with BP104 revealed that Nrg¹⁸⁰ localises in
324 the membranes of all cells from NSC lineages (Fig. 5E and Supp. Fig. 5B). In neurons,
325 it was not only found in the cell body but also in the axonal bundle, a localisation
326 reported previously [53]. In accordance with this result, *nrg* knockdown in NSC
327 lineages completely depleted the BP104 signal (Supp. Fig. 5C). We then took
328 advantage of a Nrg::GFP fusion which has been shown in other tissues to preferentially

329 target the *Nrg*¹⁶⁷ isoform (called *Nrg*¹⁶⁷::*GFP*, [48,50]). *Nrg*¹⁶⁷::*GFP* also appeared
330 enriched between cells of the same NSC lineage, where it co-localised with BP104
331 staining, except on the NSC perimeter, devoided of BP104 (Fig. 5F and Supp. Fig. 5D;
332 see dashed white line for lack of BP104). In contrast, only *Nrg*¹⁶⁷ is detected in septate
333 junctions. Taken together these data suggest that the two isoforms of *Nrg* are
334 expressed in NSC lineages.

335 While *Nrg* can also bind in an heterophilic manner, mostly homophilic interactions
336 (between same or different isoforms) have been reported. We thus wondered whether
337 an *Nrg* to *Nrg* interaction within the NSC lineages could fulfill the role of an intra-lineage
338 adhesion stronger than an CG to NSC adhesion. First, *nrg* knockdown in CG, the only
339 cell population in contact with NSC lineages (besides clonally-related cells) did not
340 recapitulate *nrg* knockdown in NSC lineages (compare Fig. 4F with Supp. Fig. 4F),
341 suggesting that homophilic *Nrg* interactions do not exist between CG and NSC
342 lineages to maintain individual encasing.

343 We then assessed the relevance of intra-lineage *Nrg* interactions in setting up a
344 differential in adhesion (Fig. 1H, panel II.2). If such adhesion is stronger than the CG
345 to NSC lineage interaction, then expressing *Nrg* in CG would force CG to interact with
346 each other (A_{L-CG} through *Nrg* would be less favoured as a higher dose of *Nrg* would
347 be present in CG due to overexpression). Strikingly, misexpressing *Nrg*¹⁸⁰ in CG from
348 larval hatching (ALH0) resulted in altered CG morphology and loss of individual
349 encasing of NSC lineages (Fig. 5G). CG membranes displayed local accumulation as
350 well as unusual curvature, and NSCs were not separated from each other by CG
351 anymore but were rather found grouped close to each other. Overexpression of *Nrg*¹⁶⁷
352 in CG (from ALH0) produced an even more dramatic phenotype, with localised,
353 compact globules of CG membranes and the complete lack of individual encasing of
354 NSC lineages (Fig. 5H). Interestingly, misexpression of a *Nrg*^{GPI} construct in which the
355 transmembrane and cytoplasmic domains are replaced by a GPI anchor signal [58]
356 also resulted in aggregated CG and clustered NSC lineages (Supp. Fig. 5E). This
357 shows that intracellular signalling through the divergent C-terminal domain is not
358 required for this sorting of CG and NSC lineages, but rather that adhesion through the
359 extracellular part mediates this effect.

360 Altogether, these results demonstrate that forcing CG to CG adhesion through *Nrg*
361 homophilic interactions is sufficient to segregate them from the whole population of
362 NSC lineages, between which weaker interactions exist. This further suggests that *Nrg*

363 homophilic adhesions between cells of the same NSC lineage are responsible for
364 keeping these cells together and excluding CG.

365 If Nrg interactions are responsible for providing binding between cells of the same NSC
366 lineage, including the stem cell, one consequence is that NSC could bind to each other.
367 That would result in several NSCs encased in the same CG chamber something we
368 do not witness in normal conditions. Interestingly, Nrg seems to be expressed in NSC
369 after their encasing (see Fig. 4B), in contrast to Nr_x-IV (Fig. 4A). This would fit with the
370 idea that early on, when NSC are not encased yet and separated from other NSCs by
371 the CG, $A_{\text{NSC-NSC}}$ is kept low. As such, a precocious expression of Nrg in NSCs would
372 be predicted to lead to their grouping (and further the grouping of their lineages) in a
373 CG chamber. Strikingly, expressing Nrg¹⁶⁷ (which gave the strongest phenotype in CG,
374 see Fig. 5G-H) from ALH0 resulted in multiple CG chambers containing several NSCs
375 (Fig. 5I-J). A similar result was obtained when expressing Nrg^{GPI} with the same timing
376 (Supp. Fig. 5F), implying that the adhesive role of Nrg is responsible for such effect.
377 This is in contrast to the lack of effect of misexpressing Wrapper in NSC lineages (also
378 from ALH0), showing that not all adhesion complexes can lead to $A_{\text{NSC-NSC}}$ high enough
379 to group NSC together. These results suggest that a proper timing in establishing intra-
380 lineage adhesion is instrumental in ensuring the individual encasing of NSC lineages
381 by CG.

382 **Nr_x-IV and Nrg adhesions are required in NSC lineages for correct axonal path** 383 **during development**

384 So far, our data show that Nr_x-IV- and Nrg-mediated adhesions in NSC lineages, while
385 likely fulfilling different roles in this process, are both important to set up the individual
386 encasing of NSC lineages by CG. We sought to assess the functional relevance of
387 such adhesions, and ultimately of individual encasing, for the cells of NSC lineages.
388 We first turned our eyes to the NSC themselves. As NSC core function is dividing to
389 produce differentiated progeny, we assessed NSC proliferation under *nrg* and *nr_x-IV*
390 knockdown in NSC lineages, using phospho-histone 3 (PH3) to mark mitotic DNA. We
391 found that both mitotic indexes and phase distribution in mitosis were similar between
392 these conditions and control (Supp. Fig. 6A-B). Of note, *shg* knockdown in NSC
393 lineages also did not lead to detectable changes in the mitotic profile (Supp. Fig. 6C).
394 These results show that Nrg and Nr_x-IV adhesion in the niche are not critical for the
395 rate of NSC proliferation

396 Improper encasing of NSC lineages during development implies that newborn
397 neuronal lineages are not physically constrained anymore nor neatly packed. Rather
398 they are expanding more freely and are found mingled with other ones. At this stage,
399 immature secondary neurons start sending axons to establish synaptic connections
400 with proper partners in the neuropile, with axons from the same lineage grouped as
401 one or two tight bundles and following the same path [27,53] (Supp. Fig. 1A and Fig.
402 6A). This axonal fascicle, also encased by the CG membrane, shows a well-defined
403 tract for each lineage, with stereotyped entry in and path within the neuropile.
404 We thus wondered whether disruption of niche adhesion and loss of lineage
405 organization could translate into an altered pattern of axonal projections. To assess
406 this possibility, we marked NSC lineages in a multicolour clonal fashion, this time using
407 a membrane version of Raeppli (CAAX tag) [34] to label both the cell body as well as
408 the extending axons (Supp. Fig. 1A and Fig. 6B-C). Both for *nrx-IV* and *nrg*
409 knockdowns, we first found that the organization in bundles of axons from neurons of
410 the same lineage appeared preserved, and that most of them still found their way to
411 the neuropile. We however noticed a less regular pattern in their path to the neuropile,
412 drifting from the classic boat shape seen from the antero-posterior view (Fig. 6A-C,
413 view 1) and appearing less aligned in a longitudinal view (Fig. 6A-C, view 2). We then
414 calculated the angle of axonal extension to the antero-posterior axis of the VNC (Fig.
415 6D). We found that, compared to a control condition at the same stage (slightly earlier
416 for *nrg* RNAI), the angles were less stereotyped in overall, with a broader distribution,
417 and slightly shifted, being either more closed (*nrx-IV* RNAi, Fig. 6E) or more open (*nrg*
418 RNAi, Fig. 6F). In addition, we also detected the rare occurrence of axonal bundles not
419 targeting the neuropile, but rather going to the edge of the organ, without establishing
420 synaptic connections (Supp. Fig. 6D). Altogether, these data show that *Nrx-IV* and *Nrg*
421 adhesions in the NSC niche both influence the extension of axonal tracts from newborn
422 neurons.

423 **The function of *Nrx-IV* and *Nrg* adhesions in NSC lineages during development** 424 **influences adult locomotor behaviour**

425 The tracts of the axonal projections established by secondary neurons during larval
426 development are mostly kept during metamorphosis, being extended and complexified
427 rather than fully remodeled [26,27]. Indeed, despite the overall change in CNS
428 morphology over time, the relative positions and pattern of tracts from different

429 lineages are maintained and recognizable [53]. As such, the correct establishment of
430 axonal projections during development is meant to be critical for the function of mature
431 neurons in the adult CNS. In this light, we decided to determine whether the loss of
432 *Nrx-IV* and *Nrg* adhesions in NSC lineages during development could impair neuronal
433 function later in the adult.

434 One way to assess neuronal function is to probe its functional output on adult
435 behaviour. As our analysis of CG and axonal tract phenotypes have focused on the
436 VNC, in which motor neurons are produced, we focused on motor parameters in the
437 adult. To do so, we took advantage of an ethoscope-based tracking system [60] to
438 record locomotion metrics. This high-throughput platform relies on video acquisition to
439 record positional data in real-time for multiple flies, individually placed in a cylindrical
440 tube of given length and volume. Several behavioural parameters can be extracted by
441 calculating the position of the fly overtime, including average locomotion speed
442 (velocity), amount of sleep (defined as the cumulative time during which a fly stay still
443 for at least five minutes, [61]) and circadian activity. Statistics on several flies finally
444 allow to draw an average behaviour for the population.

445 We recorded locomotion metrics for *shg*, *nrx-IV* and *nrg* knockdowns in NSC lineages,
446 as well as for a control line. We did it in two conditions. First, RNAi expression was
447 only allowed during larval phase, and prevented from mid-pupal stage (see Methods,
448 “induced” condition). In this case, comparing the behaviour of the different knockdowns
449 to the control line reveals the importance of each adhesion on locomotion parameters.
450 Second, gene knockdowns were never activated (same genetic background, but RNAi
451 always off; see Methods, “Non Induced” condition), a condition meant to serve as a
452 control for the effect of genetic background on locomotion parameters. We did not find
453 comparing induced and non-induced relevant, as these two conditions relies on very
454 different regimens of fly husbandry.

455 We first look at the overall pattern of activity through a circadian cycle of light and dark
456 periods. In laboratory conditions, *Drosophila* indeed displays a characteristic rest
457 (sleep)/activity pattern where they become highly active in anticipation of the
458 transitions between light and dark periods. Rest/sleep happens mostly in the middle of
459 light and dark periods. We found that this circadian pattern of activity was kept in the
460 different lines in induced condition, with two main peaks of activity (morning and

461 evening, Supp. Fig. 7A). We noticed slightly higher anticipation for the evening peak in
462 the case of *nrx-IV* and *nrg* knockdowns, as well as wider peaks for *nrg* knockdown.
463 Sleep metrics then revealed a stunning change in the behaviour of *nrg* RNAi and *nrx-*
464 *IV* RNAi flies, while *shg* RNAi appeared very similar to control (Fig. 7A, induced). While
465 control and *shg* RNAi flies were spending 70% and 68% of their time sleeping,
466 respectively, *nrg* RNAi flies spent only 27% of their time in average, a dramatic
467 reduction (Fig. 7B, induced). They appeared hyperactive throughout both light and dark
468 periods (Supp. Fig. 7B), with an especially important shift during the time between
469 activity peaks. *nrx-IV* RNAi flies also spend significantly less time sleeping, which was
470 decreased to only 58 % of their time (Fig. 7B, induced). In contrast, *shg* RNAi, *nrx-IV*
471 RNAi, *nrg* RNAi and control all behaved in a similar fashion in the non-induced
472 condition (Fig. 7A-B; ctrl = 61% ; *shg* = 53% ; *nrx-IV* = 54% ; *nrg* = 58% of time
473 sleeping).

474 We wondered whether this locomotor hyperactivity was only visible as the time flies
475 spent moving, or also in the way they were moving. We then determined the speed of
476 locomotion for the different lines. In induced conditions, we found that the mean
477 velocity throughout the cycle was increased in *nrg* (3.5) and *nrx-IV* (2.8) but not *shg*
478 (2.1) knockdowns compared to control condition (2.3). In non-induced conditions, all
479 lines exhibited similar values of velocity (ctrl = 2.0 ; *shg* = 2.2 ; *nrx-IV* = 2.4 ; *nrg* = 2.1).
480 Taken together, these results show that the functions of Nrg and and Nrx-IV, two
481 adhesion molecules required for the individual encasing of NSC lineages, are also
482 necessary during development for proper motor activity in the adult.

1 **DISCUSSION**

2 The NSC niche harbours an elaborate architecture surrounding the stem cells and their
3 growing, differentiating neuronal lineages. However, its mechanisms of formation and
4 its role on NSCs and newborn progeny remain poorly understood. Here we investigate
5 the formation of glial niches around individual NSC lineages in the *Drosophila*
6 developing CNS. Individual encasing occurs around the NSC itself, before neuronal
7 production, providing a first mechanism for implementing lineage encasing. However,
8 such timing is not the only strategy to ensure the formation and maintenance of
9 individual encasing around the entire lineage. We actually uncovered that CG are able
10 to distinguish between and sort themselves from individual NSC lineages through
11 differences in adhesion complexes, what provides a belt and braces mechanism to
12 ensure lineage encasing regardless of timing. Several components of both adherens
13 and occluding junctions are indeed expressed in NSC lineages. While adherens
14 junctions appear mostly dispensible for lineage encasing, two SJ components, Nr_x-IV
15 and Nrg, are required for this structure, however outside of their junctional roles. Nr_x-
16 IV binds to Wrapper present on the CG, and Nrg, expressed after neuronal production
17 starts, performs homophilic interactions with itself to bind cells from one lineage
18 together. This Nrg-based intra-lineage adhesion is instrumental in sorting NSC lineage
19 and CG after neuronal production, providing a stronger adhesion compared to the Nr_x-
20 IV to Wrapper interaction. Finally, we found removing Nr_x-IV and Nrg during larval
21 stage leads to behavioural defects in adult, producing hyperactive flies. Altogether, our
22 findings show that a timely difference in adhesion between NSC/NSC lineages and
23 niche cells defines the structure of the niche during development and influences adult
24 behaviour (Fig. 7D).

25 Both adherens and occluding junctions are associated with and as such mostly have
26 been mostly described in epithelia and epithelial-like tissues. Here the fact that core
27 components of adherens (E-cadherin, β -catenin) and occluding (Nr_x-IX, Nrg, Dlg1,
28 ATPa, Cora) junctions localise in stem cell and maturing progeny raises questions
29 about their regulation and role in such cell types. While adherens junction appears to
30 be specifically set up in NSC lineages, we did not find them functionally relevant for
31 individual encasing by CG, NSC proliferation and motor behaviour in adult. Previous
32 studies had reported that E-cadherin disruption was leading to defects in CG
33 architecture and altering NSC proliferation. However, both studies used a dominant-

34 negative form of E-cadherin [22,41]. Here, we could not recapitulate such
35 consequences using an efficient RNAi knockdown nor a null allele of E-cadherin (Fig.
36 3C-D and Supp. Fig. 3C, G). It is possible that some of the effects observed previously
37 are neomorphic and triggered by the activation of other pathways. Another possibility
38 is the fact that knockdown, but not competition by a dominant-negative, could lead to
39 compensation (such as an increase in N-cadherin), masking the role of E-cadherin and
40 adherens junction. Nevertheless, while we believe adherens junctions are not
41 instrumental in establishing CG architecture around NSC lineages, it might be used as
42 a strengthening, safety mechanism, available when other processes fail. In this light, it
43 would be relevant to determine whether *shg* knockdown potentializes *nrg* knockdown.
44 Other roles in NSC lineages, possibly subtle, also await to be uncovered.

45 We propose that a balance between a strong Nrg-based adhesion within the NSC
46 lineages and a weaker, Nr_x-IV-based interaction between CG and NSC lineages (A_{L-}
47 $C_G < A_L$) builds the stereotyped, individual encasing of NSC lineages. There is no direct
48 measure of the strength of adhesion between Nr_x-IV and Wrapper compared to Nrg with
49 itself. However, the fact that misexpressing Nrg in CG creates CG aggregates and
50 alters individual encapsulation indicates that Nrg can surpass the endogenous Nr_x-IV
51 to Wrapper interaction. In this line, misexpressing Wrapper in the NSC lineages does
52 not alter encasing, suggesting that increasing A_L compared to A_{L-C_G} does not change
53 the directionality of the difference, and as such that this difference is already there.
54 Similarly, E-cadherin misexpression in the CG has no impact on lineage encasing,
55 implying that the sum of the adhesions present in the NSC lineages outweighs the
56 presence of *de novo* adherens junctions between lineages and CG.

57 Nr_x-IV interaction with Wrapper could be important to provide a scaffold onto which
58 anchoring the glial membrane on the available surface of all lineage cells. When this
59 scaffold is weakened, CG randomly infiltrate in between NSC lineages still tightly
60 bound by Nrg interaction, leading to the creation of CG chambers of variable size (Fig.
61 4D-E). Such chambers appear neat, with a clear outline around grouped lineages and
62 the absence of CG membrane signal within. Such striking, unmistakable phenotype,
63 which we never observed previously, suggests that upon alteration of Nr_x-IV and
64 Wrapper interaction, CG still recognize NSC lineages as “wholes”, but cannot
65 implement their individual encapsulation.

66 We propose that Nrg interact with itself in NSC lineages. We found that the Nrg¹⁸⁰
67 isoform was expressed in newborn secondary neuron, in accordance with its known
68 neuronal association in other life stages. However, we propose that Nrg¹⁶⁷, traditionally
69 associated with junctional localisation, is also present. This is based on the use of a
70 Nrg::GFP fusion shown to preferentially target the Nrg¹⁶⁷ isoform. We also noticed that
71 driving *nrg* RNAi in NSC lineages, under the same condition, completely abolished the
72 signal from Nrg¹⁸⁰ (BP104, Supp. Fig. 5C), but not from the *Nrg::GFP* fusion (Supp.
73 Fig. 4D). Whether Nrg¹⁶⁷ interacts with Nrg¹⁸⁰ for NSC encasing, or has another
74 junctional role, remains to be demonstrated. Of note, an elegant study has shown that
75 neuronal Nrg¹⁸⁰ can bind to epidermal Nrg¹⁶⁷ to prevent homologous Nrg¹⁸⁰-mediated
76 dendrite bundling and effectively promote enclosure of single-neuron dendrites by the
77 epidermis [50], showing that isoform interactions exist to mediate cell-cell adhesion.
78 The temporal regulation of Nrg expression appears crucial. We found it is not present
79 in NSCs before encapsulation (Fig. 4B), whereas its precocious expression results in
80 NSC lineages grouped together (Fig. 5I-J and Supp. Fig. 5F). What triggers this timely
81 change, and especially its link with NSC reactivating, is an intriguing question. Indeed,
82 what first recruits CG membrane to NSC, before creating and clustering a lineage,
83 remain to be identified.

84 The phenotypic expressivity of *nrg* knockdown in NSC lineages is variable, with a
85 minority showing restricted defects, an observation we do not explain. Nevertheless,
86 in most larvae, we find that *nrg* knockdown in NSC lineages result in multiple CG
87 chambers with several NSC lineages grouped together, and in half of the case with
88 only very few individual encasings left (Fig. 4H). In these conditions, cells from a same
89 lineage still appear to be mostly kept together, as a group. If Nrg binds cells from the
90 same lineage together, a potential outcome of its loss of function could have been for
91 these cells to end up individually encapsulated, something we do not see. However,
92 the existence of other adhesion complexes (such as E-cadherin) still providing
93 cohesion might be enough to prevent the case where $A_L < A_{L-CG}$ even under *nrg*
94 knockdown. Ultimately, the total sum of adhesions for each cell pair decides of the
95 directionality of the difference. We also do not know the adhesion strength between
96 CG cells. If it is higher than the sum of remaining adhesions in NSC lineages after *nrg*
97 knockdown, it could explain the fact that CG do not extend to separate NSC lineages.

98 The loss of *Nrx-IV* and *Nrg* adhesions both result in altered axonal tracts coming from
99 the newborn neurons. Although it could be a consequence of some autonomous
100 properties of these molecules on neuronal/axonal features, previous studies had
101 already linked a change in CG structure or function to misshaped axonal tracts in the
102 larval CNS, both in the larval optic lobe [17] or in the central brain, where genetic
103 ablation of CG results in abnormal axonal trajectories and fasciculation [28].
104 We further linked the loss of *Nrx-IV* and *Nrg* adhesions during development to changes
105 in the locomotor behaviour of the resulting adults, which appeared hyperactive. While
106 *Nrg* could be involved in other ways than through building the niche structure, due its
107 homotypic interactions between neurons and its strong axonal localization, *Nrx-IV* and
108 *Wrapper* interaction makes a stronger case for bridging niche architecture with adult
109 behaviour. First, *Nrx-IV* to *Wrapper* interaction is between lineages and CG, rather
110 than between neurons. Moreover, in *nrx-IV* knockdown in NSC lineages and *wrapper*
111 knockdown in CG, CG chambers are still present, neatly delineated around multiple
112 NSC lineages, depicting the loss of the individuality of encasing rather than a
113 comprehensive alteration of CG structure. This pleads for an impact of a targeted,
114 specific remodelling of niche architecture during development on adult behaviour.
115 Hyperactivity (increased locomotor activity and reduced sleep) in *Drosophila* has been
116 found in diverse models of neuro/developmental and neurological disorders, including
117 the Fragile X syndrome [62], Attention-deficit/hyperactivity disorder (ADHD) [63] and
118 Shwachman–Diamond syndrome [64]. In all these cases, changes in locomotion were
119 correlated with synaptic and axonal abnormalities. Hyperactivity also appears during
120 starvation, under hormonal control [65,66]. In our case, a reasonable explanation
121 would be that axonal misprojection following loss of adhesion in the neurogenic niche
122 during development translates to dysfunctional motor neurons in the adult.

123 Here, we propose a mechanism in which the temporal and spatial localization of
124 different adhesion complexes results in the formation of a stereotypic niche organizing
125 individual NSC lineages and their progeny. Their function is also important for axonal
126 projection of newborn neurons, and locomotor behaviour in the adult, thus linking niche
127 adhesive properties and developmental neurogenesis to adult health. All these
128 complexes are heavily conserved in mammals, warranting the question of their non-
129 junctional role in a developing CNS.

130 **METHODS**

131 **Methods**

132 **Fly lines and husbandry**

133 *Drosophila melanogaster* lines were raised on standard cornmeal food at 25°C. Lines
 134 used in this study are listed in the table below:

Strains	Source	Stock number/Reference
<i>w¹¹¹⁸</i>	BDSC	5905
<i>Nervana2::GFP (Nrv2::GFP)</i>	BDSC	6828
<i>Shg::GFP</i>	Yohanns Bellaïche lab	
<i>Nrx-IV::GFP</i>	Christian Klämbt lab	<i>Nrx⁴⁵⁴</i> , [67]
<i>Nrg::GFP</i>	Kyoto	110658
<i>Nrg¹⁶⁷::GFP</i>	BDSC	6844
<i>Dlg1::GFP</i>	BDSC	50859
<i>ATPalpha::GFP (CPTI)</i>	Kyoto DGGR	115323
<i>tubulin-GAL80^{thermosensitive(ts)}</i>	BDSC	65406
<i>yw, hs-FLP</i>	Andrea Brand lab	
<i>FRT G13</i>	BDSC	1956
<i>CoinFLP</i>	BDSC	58750
<i>cyp4g15-GAL4</i>	BDSC	39103
<i>cyp4g15-FRT-STOP-FRT-GAL4</i>	This study	
<i>cyp4g15-FRT-STOP-FRT-LexA</i>	Spéder lab	[25]
<i>cyp4g15-FLP</i>	Spéder lab	[25]
<i>cyp4g15-mtd::Tomato</i>	Spéder lab	[25]
<i>wor-Gal4</i>	BDSC (Doe lab insertions)	56553 & 56554
<i>PntP1-GAL4</i>	Jan lab	[68]
<i>CRIMIC wrapper</i>	BDSC	93483
<i>cyp4g15-QF2</i>	Spéder lab	
<i>tub-QS</i>	BDSC	52112
<i>TUG G13 MARCM line</i> <i>y,w, hs-FLP; FRTG13, tubP-GAL80[LL2]/</i> <i>(CyO, act-GFP[JMR1]); tubP-GAL4[LL7],</i> <i>UAS-mCD8-GFP[LL6]/TM6B</i>	Bruno Bello	
<i>tubP-GAL80[LL2]</i>	BDSC	5140
<i>UAS-reaper</i>	Andrea Brand lab	
<i>UAS-mCD8::GFP</i>	BDSC	5130
<i>UAS-mCD8::RFP</i>	BDSC	27399
<i>UAS-Raepli CAAX 43E</i>	Generated from BDSC 55082	[25]
<i>UAS-Raepli NLS 53D</i>	Generated from BDSC 55087	[25]
<i>UAS-Raepli NLS 89A</i>	Generated from BDSC 55088	[25]
<i>UAS-prospéro RNAi</i>	Andrea Brand lab	
<i>UAS-brat RNAi</i>	Andrea Brand lab	

<i>UAS-aPKc</i>		[69]
<i>UAS-shg RNAi</i>	BDSC	32904
	VDRC	27082
	VDRC	103962
<i>UAS-arm RNAi</i>	VDRC	27227
<i>UAS-nrx IV RNAi</i>	BDSC	32424
<i>UAS-nrg RNAi</i>	BDSC	37496
<i>UAS-wrapper RNAi</i>	BDSC	29561
<i>UAS-ATPα RNAi</i>	BDSC	28073
<i>UAS-nrg¹⁸⁰</i>	BDSC	24169
<i>UAS-nrg¹⁶⁷</i>	BDSC	24172
<i>UAS-nrg^{GPI}</i>	BDSC	24168
<i>UAS-wrapper</i>	BDSC	78535
<i>QUAS-PTEN</i>	This study	
<i>shg^{null} (shg^{R64a})</i>	Yohanns Bellaïche lab	

135

136 Larval culture and staging

137 Embryos were collected within 2-4 hours window on grape juice-agar plates and kept
138 at 25°C for 20-24 hours. Freshly hatched larvae were collected within a 1 hour time
139 window (defined as 0 hours after larval hatching, ALH0), transferred to fresh yeast
140 paste on a standard cornmeal food plate and staged to late first instar (ALH24), late
141 second instar (ALH48), mid third instar (ALH72) and late third instar (ALH96).

142 For growth on quinic acid, food plates were prepared by mixing 250 mg/ml stock
143 solution of quinic acid (dissolved in sterile water) into melted food at 50°C, for a final
144 concentration of 20 mg/ml of quinic acid.

145

146 DNA cloning and *Drosophila* transgenics

147 A portion of the *cyp4g15* enhancer (GMR55B12, Flybase ID FBsf0000165617), which
148 drives in the cortex glia and (some) astrocyte-like glia, was amplified from genomic
149 DNA extracted from *cyp4g15*-GAL4 adult flies, with a minimal *Drosophila* synthetic
150 core promoter [DSCP] [70] fused in C-terminal. For creating *cyp4g15*-FRT-STOP-FRT-
151 GAL4, a FRT STOP cassette was amplified from an UAS-FRT.STOP-Bxb1 plasmid
152 (gift from MK. Mazouni) and the GAL4 sequence was amplified from the entry vector
153 pENTR L2-GAL4::p65-L5 (gift from M. Landgraf). The two amplicons were joined
154 together by overlapping PCRs. This FRT-STOP-FRT-GAL4 amplicon together with the
155 *cyp4g15*^{DSCP} enhancer were inserted in the destination vector pDESThaw sv40 using
156 Multisite gateway system [71] to generate a *cyp4g15*^{DSCP}-FRT-STOP-FRT-GAL4

157 construct. The construct was integrated in the fly genome at an attP2 or attP40 docking
158 sites through PhiC31 integrase-mediated transgenesis (BestGene). Several
159 independent transgenic lines were generated and tested, and one was kept for each
160 docking site.

161 For creating *QUAS-PTEN*, the *PTEN* coding sequence was amplified from genomic
162 DNA extracted from *UAS-PTEN* [72] adult flies, as described in [73]. This amplicon
163 together with the QUAS sequence (pENTRY L1-QUAS-R5, gift from S.Stowers) were
164 joined using the Multisite gateway system [71] in the destination vector pDESThaw
165 sv40 gift from S. Stowers). The construct was integrated in the fly genome at an attP40
166 docking site through PhiC31 integrase-mediated transgenesis (BestGene). Several
167 independent transgenic lines were generated and tested, and one was kept (*QUAS-*
168 *PTEN*).

169

170 **Fixed tissue Immunohistochemistry and imaging**

171 For immunohistochemistry, CNS from staged larvae were dissected in PBS, fixed for
172 20 min in 4% formaldehyde diluted in PBS, washed three times in PBS-T (PBS+0.3%
173 Triton X-100) and incubated two nights at 4°C with primary antibodies diluted in
174 blocking solution (PBS-T, 5% Bovine Serum Albine, 2% Normal Goat Serum). After
175 washing three times in PBS-T, CNS were incubated overnight at 4°C or 3-3 h at room
176 temperature with secondary antibodies (dilution 1:200) diluted in blocking solution.
177 Brains were washed three times in PBS-T and mounted in Mowiol mounting medium
178 on a borosilicate glass slide (number 1.5; VWR International). For the NrX-IV antibody,
179 CNS were fixed for 3 minutes in Bouin's fixative solution (Sigma Aldrich, HT10132),
180 and the rest of the protocol was identical. Primary antibodies used were: guinea pig
181 anti-Dpn (1:5000,[25]), chicken anti-GFP (1:2000, Abcam ab13970), rat anti-ELAV
182 (1:100, 7E8A10-c, DSHB), mouse anti-ELAV (1:100, 9F8A9-c, DSHB), rat anti-dE-
183 cadherin (1:50, DCAD2, DSHB), mouse anti-Armadillo (1:50, N2 7A1, DSHB), rabbit
184 anti-Repo (1:10000, kind gift from B. Altenheim), mouse anti-Repo 1:100 (DSHB,
185 8D12-c), mouse anti-Prospero (1:100, MR1A, DSHB), rabbit anti-Asense (1:3000, kind
186 gift from the Yan lab). rabbit anti-Phospho-histone H3 (1:100, Millipore 06-570), rabbit
187 anti-Nrx-IV (1:1000, [46]) mouse anti-wrapper (1:20, DSHB 10D3, supernatant),
188 mouse anti-Nrg¹⁸⁰ (1:50, DSHB BP104, supernatant). Fluorescently-conjugated
189 secondary antibodies Alexa Fluor 405, Alexa Fluor 488, Alexa Fluor 546 and Alexa
190 Fluor 633 (ThermoFisher Scientific) were used at a 1:200 dilution. DAPI (4',6-

191 diamidino-2-phenylindole, ThermoFisher Scientific 62247) was used to counterstain
192 the nuclei.

193

194 **Image acquisition and processing**

195 Confocal images were acquired using a laser scanning confocal microscope (Zeiss
196 LSM 880, Zen software (2012 S4)) with a Plan-Apochromat 40x/1.3 Oil objective. All
197 brains were imaged as z-stacks with each section corresponding to 0.3-0.5 μm . The
198 spectral mode was used for acquiring pictures of Raeppli clones. Images were
199 subsequently analysed and processed using Fiji (Schindelin, J. 2012), Volocity (6.3
200 Quorum technologies), and the Open-Source software Icy v2.1.4.0 (Institut Pasteur
201 and France Bioimaging, license GPLv3). Denoising was used for some images using
202 the Remove noise function (Fine filter) in Volocity. Images were assembled using
203 Adobe Illustrator 25.4.6.

204

205 **Multicolour clonal analyses (Raeppli)**

206 Raeppli clones were generated by subjected freshly hatched larvae (ALH0) to a 37°C
207 heat shocked for 2 hours. The genetic crosses and culture conditions were the
208 following:

209 • Fig. 1G:

210 *yw, hs-FLP ; Nrv2::GFP, wor-GAL4/CyO*

211 *x UAS-Raeppli-NLS 53D; UAS-pros RNAi, tubGAL80^{ts}*

212 *x UAS-Raeppli-NLS 53D; UAS-brat RNAi, tubGAL80^{ts}*

213 *x UAS-Raeppli-NLS 53D; tubGAL80^{ts}*

214 For *brat* RNAi, larvae were subjected to a 2 h heatshock at 37°C just after larval
215 hatching (ALH0), then transferred to 29°C.

216 For *pros* RNAi, larvae were kept at 18°C for 48h after collection and then subjected to
217 a 2 h heatshock at 37°C. The larvae were transferred to 29°C afterwards.

218 • Fig. 2B, 3E and Supp. Fig. 2B, 3F-G:

219 *tub-QS; Nrv2::GFP, wor-GAL4/CyO*

220 *x yw, hs-FLP; UAS-Raeppli-NLS 53D*

221 *x yw, hs-FLP; UAS-Raeppli- NLS 53D, QUAS-PTEN*

222 *x yw, hs-FLP; UAS-Raeppli- NLS 53D, QUAS-PTEN; UAS-shg RNAi VDRC 27082*

223 72h at 29°C on plates with 20 mg/ml quinic acid (T1) followed by 28h at 29°C on plates
224 without quinic acid (T2)

225 • Fig. 3D:

226 *Nrv2::GFP; wor-GAL4/CyO;*

227 *x yw, hs-FLP; UAS-Raeppli-nls 53D; UAS-shg RNAi VDRC 27082*

228 Larvae were kept 68h at 29°C from ALH0.

229 • Fig. 4D:

230 *Nrv2::GFP; wor-GAL4/CyO; UAS-Raeppli-nls 89A*

231 *x yw, hs-FLP; UAS-Nrx-IV RNAi*

232 Larvae were kept 72h at 29°C from ALH0.

233 • Fig. 4G :

234 *yw, hs-FLP; Nrv2::GFP; wor-GAL4/CyO; tub-Gal80^{ts}*

235 *x UAS-nrg RNAi ; UAS-Raeppli-NLS 89A*

236 Just hatched larvae (ALH0) were subjected to a 2 h heatshock at 37°C and then kept
237 at 18°C for 24 h after collection. The larvae were transferred to 29°C afterwards, and
238 dissected 54 h later.

239 • Fig. 6B:

240 *yw, hs-FLP; Nrv2::GFP; wor-GAL4/CyO; tub-Gal80^{ts}*

241 *x UAS-Raeppli-CAAX 42D*

242 *x UAS-Raeppli-CAAX 42D ; UAS-Nrx-IV RNAi*

243 Just hatched larvae (ALH0) were subjected to a 2 h heatshock at 37°C, transferred to
244 29°C afterwards, and dissected 72 h later.

245 •• Fig. 6C:

246 *yw, hs-FLP ; Nrv2::GFP, wor-GAL4/CyO; tub-GAL80^{ts}*

247 *x UAS-Raeppli-CAAX 99E*

248 *x UAS-nrg RNAi, UAS-Raeppli-CAAX 99E*

249 Just hatched larvae (ALH0) were subjected to a 2 h heatshock at 37°C and then kept
250 at 18°C for 24 h after collection. The larvae were transferred to 29°C afterwards, and
251 dissected 54 h later.

252

253 ***shg^{null}* MARCM clones**

254 *shg^{64R}, FRT42B; cyp4g15-myr::dTomato* flies were crossed to the TUG13 MARCM line
255 (*y,w, hs-FLP ; FRTG13, tubP-GAL80[LL2]/ (CyO, act-GFP[JMR1]) ; tubP-GAL4[LL7],*
256 *UAS-mCD8-GFP[LL6]/TM6B*). The resulting progeny was let to develop at 25°C, then
257 subjected to 37°C heatshock either at 14-18 h after egg laying for 2 h, or at ALH48 for
258 30 min, and finally dissected at ALH72 (Supp. Fig. 3C).

259 **Clonal analyses using CoinFLP**

260 The Coin-FLP method [36] was used to induce rare clones of PTEN expressing CG
261 cells, by crossing *cyp4g15-FLP* ; *CoinFLP GAL4::LexA* ; *UAS-mCD8::RFP* females to
262 to *UAS-PTEN* males or *w1118* males for control, and maintained at 25°C. Larvae were
263 staged to ALH48-ALH72 at 25°C.

264

265 **Quantification of NSC lineage encasing (Raeppli-NLS)**

266 For each VNC the total number of chambers containing more than one NSC, and the
267 corresponding total number of NSC lineages non-individually encased were
268 determined by counting manually the number of different Raeppli clones (colours)
269 contained within one continuous CG membrane, choosing the z plane where
270 differentiating lineages were well visible (at mid-distance between the CNS surface and
271 the neuropile).

272

273 **Quantification of NSC mitotic index**

274 CNSs of the chosen genotypes were stained with Dpn and phospho-histone H3
275 antibodies to detect NSC fate and mitosis, respectively. Quantification was performed
276 on NSCs from the thoracic part of the VNC. Mitotic phases (Prophase,
277 Prometaphase/metaphase, Anaphase, Telophase) were manually determined by the
278 localization and pattern of PH3⁺ DNA and Dpn staining. Normalized mitotic index
279 corresponds to the ratio between mitotic NSCs over all NSCs, then divided by the
280 mean of this ratio for the control sample.

281

282 **Quantification of axonal angle**

283 Z stacks of Raeppli CAAX clones induced in NSC lineages were visualized in Volocity
284 (6.3 Quorum technologies), in a lateral view along the antero-posterior axis (xz axis).
285 The brightest colour (mTFP1) was chosen and lines were drawn parallel to and
286 following the main axonal projection for each clone. Pictures of the line were recorded
287 through snapshots, and imported into Icy v2.1.4.0 where the Angle Helper plugin was
288 used to measure the angle formed with the intersection with the AP axis (Fig. 6D-F).

289

290 **Behavioural analysis**

291 Locomotor activity of individual flies was measured with the *Drosophila* ethoscope
292 activity Open Source system [60] at 23°C. Young adult males (7-10 days) were

293 individually placed in 6.5 cm transparent tubes containing 1.5 cm nutrient medium
294 (agarose 2% (p/v), sucrose 5% (p/v)) closed with wax at one end and cotton at the
295 other. 20 tubes were positioned in each ethoscope and flies were first entrained to
296 12hr:12hr light-dark (LD) cycles for 3 days and their activity was recorded for 3 more
297 days. The activity data analysis was done with the R software, using Rethomics
298 packages [74]. Flies were considered asleep when found motionless for at least 5 min.
299 The percentage of time sleeping across LD cycles was measured as the fraction of
300 time sleep within 30 min intervals, whereas global time sleeping per fly corresponds to
301 a total sleep over total time ratio. Velocity was measured using a previously described
302 tracking algorithm [74] and is expressed in relative units. Fly moving across LD cycles
303 was calculated as the fraction of time moving within 30 min time intervals. Overall, 35
304 control (non induced) flies and 55 (induced) flies, coming from 8 independent
305 experiments, were analysed for each genotype. Genotypes positions in 20 tubes
306 arenas were changed from one experiment to another to avoid positional bias.

307

308 **Statistics and reproducibility**

309 Statistical tests used for each experiment are stated in the figure legends. Statistical
310 tests were performed using GraphPad Prism 7.0a. For all box and whisker plots,
311 whiskers mark the minimum and maximum, the box includes the 25th–75th percentile,
312 and the line in the box is the median. Individual values are superimposed.

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12 **Author Contributions**

13 A.B-L, P.S., V.R and I.G. conceived the experimental design. A.B-L. performed
14 experiments of Figs. 1; 2; 3A-C, F-G and Supp. Figs. 1B-C; 2; 3A-B, H. P.S. performed
15 experiments of Figs. 3D, H; 4C-H; 5; 6 and Supp. Fig. 3C,D,G; 44; 5; 6. V.R. performed
16 experiments of Figs. 7A-C and Supp. Fig. 7. D.B. performed experiments of Fig 4A-B.
17 A.B-L., V.R. and P.S. analyzed the data. A.B-L., V.R. and P.S. wrote the manuscript.

18 **Declaration of Interests**

19 The authors declare no competing interest.

20 **Data availability statement**

21 The datasets generated during and/or analysed during the current study are available
22 from the corresponding author on reasonable request.

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1 **Figure legends Banach-Latapy et al.**

2

3 **Figure 1. Cortex glia recognize lineage over identity to generate stereotypic**
4 **encasing of NSC lineages.**

5 A) Schematic of the *Drosophila* larval CNS depicting the localisation of the NSC
6 lineages. Two main neurogenic regions are the central brain (CB), comprising two
7 hemispheres, and the ventral nerve cord (VNC).

8 B) Schematic of the Type I and Type II NSC lineages.

9 C) Confocal pictures representing the dorsal region of the larval CB and ventral region
10 of the VNC at ALH72 (at 25°C) labelled with markers for the CG membranes
11 (*Nrv2::GFP*, green), glia nuclei (anti-Repo, yellow), NSC (anti-Dpn, grey) and neurons
12 (anti-ElaV, magenta).

13 D) Schematic of the NSC niche, made by the perineurial glia (PG, brown),
14 subperineurial glia (SPG, orange), cortex glia (CG, green), neural stem cells (NSC,
15 grey), ganglion mother cells/intermediate progenitors (gmc/inp, blue) and neurons (N,
16 magenta).

17 E) Timeline of the encasing of NSC lineages by CG parallel to NSC behaviour.

18 F) Adaptation of the CG network to NSC tumours. Type I (*wor-GAL4 > pros RNAi*) and
19 Type II (*PntP1-GAL4 > brat RNAi*) tumours were induced and the organisation of CG
20 membrane was monitored by *Nrv2::GFP* (green). NSCs are labelled with Dpn (grey),
21 and neuron with ElaV (magenta). See Methods for timing, conditions and genetics of
22 larval rearing.

23 G) Relationship between individual encasing and cell identity. Type I (*wor-GAL4 > pros*
24 *RNAi*) and Type II (*PntP1-GAL4 > brat RNAi*) tumours were induced together with the
25 multicolour lineage tracing Raeppli-NLS. One out of four colours (blue, white, orange
26 and red) is stochastically chosen in the transformed NSC upon induction Heat shock
27 induction for 2 h at 37°C was performed at ALH0 using *hs-Flp*. CG membrane is
28 visualized with *Nrv2::GFP* (green). See Methods for timing, conditions and genetics of
29 larval rearing.

30 H) Schematic of the different hypotheses explaining individual encasing of NSC
31 lineages by CG. Panel I depicts the NSC-driven timing of NSC encapsulation, prior to
32 lineage generation, as the instructive cue. Panel II describes the use of adhesion
33 mechanisms: 1. Specific CG to NSC lineage and 2. Generic, based on difference in

34 strength between intra-lineage adhesion (A_L) and adhesion linking NSC lineages and
35 CG (A_{L-CG}); in this case, the prediction is that $A_L > A_{L-CG}$.

36

37 **Figure 2. Cortex glia use intrinsic and generic NSC lineage cues for individual**
38 **encasing.**

39 A) Schematic of the timing and genetic conditions used to probe the importance of
40 NSC-driven timing of NSC encapsulation in keeping NSC lineages together. CG
41 growth is initially blocked using PTEN expression in the CG. At T1, the block is
42 removed, and CG structure is assessed at T2.

43 B) Representative confocal picture of the extent of individual encasing of NSC lineages
44 by CG after the regimen described in A. Top panel shows the whole thoracic VNC, and
45 bottom panel a close-up of the yellow box. NSC lineages were marked with the
46 multicolour lineage tracing Raeppli-NLS (blue, white, orange and red), induced at
47 ALH0 using *hs-Flp*. CG membrane is visualized with *Nrv2::GFP* (green). See Methods
48 for timing, conditions and genetics of larval rearing.

49 C) Quantification of the number of NSC lineages non-individually encased from B).
50 Control (n = 8 VNCs) and PTEN conditional block (n = 5 VNCs). Data statistics: Mann-
51 Whitney test. Results are presented as box and whisker plots.

52 D) Schematic of the experiment designed to probe whether specific, non-
53 interchangeable adhesions exist between individual CG cells and individual NSC
54 lineages.

55 E) Representative confocal picture of the CG network in control CG clone and in clone
56 in which CG growth was blocked (PTEN). The CoinFLP system was used to generate
57 wild-type and PTEN clones in the CG. See Methods for timing, conditions and genetics
58 of larval rearing. The membrane of the clone is marked by mCD8::RFP (magenta) CG
59 membrane is visualized with *Nrv2::GFP* (green).

60

61 **Figure 3. Intra-lineage adherens junctions are not absolutely required for**
62 **individual encasing while providing robustness.**

63 A) Representative confocal images of the expression of the *Drosophila* E-cadherin,
64 Shg, at ALH0, ALH24, ALH48 and ALH72 at 25°C. Shg is monitored through a
65 *Shg::GFP* fusion (magenta), CG membrane is visualized by *Nrv2::GFP* (green), NSCs
66 are labelled with Dpn (grey), and neurons are labelled with ElaV (blue).

67 B) Representative confocal images of the expression of the *Drosophila* β -catenin, Arm,
68 at ALH72 at 25°C. Arm is detected with a specific antibody (magenta), CG membrane
69 is visualized by *Nrv2::GFP* (green), NSCs are labelled with Dpn (grey), and neurons
70 are labelled with ElaV (blue).

71 C) Representative confocal pictures of the thoracic VNC for control and *shg*
72 knockdown by RNAi in NSC lineages ($L^{NSC} > shg\ RNAi$, driver line *Nrv2::GFP*, *wor-*
73 *GAL4*; *tub-GAL80^{ts}*). Larvae are dissected after 68 h at 29°C from ALH0. CG
74 membrane is visualized by *Nrv2::GFP* (green) and NSCs are labelled with Dpn (grey).

75 D) Representative confocal picture of the thoracic VNC for *shg* knockdown by RNAi in
76 NSCs marked with the multicolour lineage tracing Raeppli-NLS (blue, white, orange
77 and red-. Raeppli-NLS is induced at ALH0 using *hs-Flp*. Larvae are dissected after
78 68 h at 29°C from ALH0. CG membrane is visualized with *Nrv2::GFP* (green). See
79 Methods for timing, conditions and genetics of larval rearing.

80 E) Schematic of the timing and genetic conditions used to probe the importance of Shg
81 adhesion (*shg* RNAi) on individual encasing of NSC lineages when CG growth is
82 initially blocked (PTEN). At T1, the PTEN block is removed, and CG structure is
83 assessed at T2.

84 F) Representative confocal picture of the extent of individual encasing of NSC lineages
85 by CG after the regimen described in E). Top panel shows the whole thoracic VNC,
86 and bottom panel a close-up of the yellow box. Dashed white lines highlight NSC
87 clones encased together. NSC lineages were marked with the multicolour lineage
88 tracing Raeppli-NLS (blue, white, orange and red), induced at ALH0 using *hs-Flp*. CG
89 membrane was visualized with *Nrv2::GFP* (green). See Methods for timing, conditions
90 and genetics of larval rearing.

91 G) Quantification of the number of NSC lineages non-individually encased from F).
92 Control (n = 8 VNCs), PTEN conditional block (n = 5 VNCs) and PTEN conditional
93 block + *shg* RNAi (n = 10 VNCs). Data statistics: one-way ANOVA with a Kruskal–
94 Wallis multiple comparison test. Results are presented as box and whisker plots.

95 H) Representative confocal picture of the thoracic VNC and close-up for *shg*
96 overexpression in the CG (driver *Nrv2::GFP*, *tub-GAL80^{ts}*; *cyp4g15-GAL4*). Larvae are
97 dissected after 68 h at 29°C from ALH0. CG membrane is visualized by *Nrv2::GFP*
98 (green), NSCs are labelled with Dpn (grey), neurons are labelled with ElaV (blue) and
99 Shg is detected with a specific antibody (magenta).

101 **Figure 4. Neurexin-IV and Neuroglian are both required in NSC lineages for**
102 **individual encasing.**

103 A) Representative confocal images of the expression of Neurexin-IV (Nrx-IV) at ALH0,
104 ALH24, ALH48 and ALH72 at 25°C. Nrx-IV is monitored through a *Nrx-IV::GFP* fusion
105 (magenta), CG membrane is visualized by *Nrv2::GFP* (green), NSCs are labelled with
106 Dpn (grey), and neurons are labelled with ElaV (blue).

107 B) Representative confocal images of the expression of Neuroglian (Nrg) at ALH0,
108 ALH24, ALH48 and ALH72 at 25°C. Nrg is monitored through a *Nrg::GFP* fusion
109 (magenta), CG membrane is visualized by *Nrv2::GFP* (green), NSCs are labelled with
110 Dpn (grey), and neurons are labelled with ElaV (blue).

111 C) Representative confocal picture (median and orthogonal views) of the thoracic VNC
112 for a condition in which *nrx-IV* is knocked down by RNAi from ALH0 in NSC lineages
113 ($L^{NSC} > nrx-IV \text{ RNAi}$, driver line *Nrv2::GFP*, *wor-GAL4*; *tub-GAL80^{ts}*). Larvae are
114 dissected after 68 h at 29°C. CG membrane is visualized by *Nrv2::GFP* (green) and
115 NSCs are labelled with Dpn (grey). The dashed white line on the orthogonal view
116 indicates the plane chosen for the median view.

117 D) Representative confocal picture of the thoracic VNC for a condition in which *nrx-IV*
118 is knocked down by RNAi from ALH0 in NSC lineages marked with the multicolour
119 lineage tracing Raepli-NLS (blue, white, orange and red). Raepli-NLS is induced at
120 ALH0 using *hs-Flp*. CG membrane was visualized with *Nrv2::GFP* (green). See
121 Methods for timing, conditions and genetics of larval rearing.

122 E) Quantification of the number of NSC lineages non-individually encased from D).
123 Control (n = 7 VNCs) and *nrx-IV RNAi* (n = 11 VNCs). Data statistics: Mann-Whitney
124 test. Results are presented as box and whisker plots.

125 F) Representative confocal picture (median and orthogonal views) of the thoracic VNC
126 for a condition in which *nrg* is knocked down by RNAi in NSC lineages ($L^{NSC} > nrg$
127 *RNAi*, driver line *Nrv2::GFP*, *wor-GAL4*; *tub-GAL80^{ts}*). Larvae are dissected after 24 h
128 at 18°C followed by 54 h at 29°C. CG membrane is visualized by *Nrv2::GFP* (green)
129 and NSCs are labelled with Dpn (grey). The dashed white line on the orthogonal view
130 indicates the plane chosen for the median view. This phenotype is seen in 7/10 cases,
131 3/10 show a milder phenotype.

132 G) Representative confocal pictures of the thoracic VNC for a condition in which *nrg* is
133 knocked down by RNAi in NSC lineages marked with the multicolour lineage tracing
134 Raepli-NLS (blue, white, orange and red). Raepli-NLS is induced at ALH0 using *hs-*

135 *Flp*, and RNAi after 24 h at 18°C. Larvae are dissected 54 h after RNAi induction. CG
136 membrane was visualized with *Nrv2::GFP* (green). See Methods for timing, conditions
137 and genetics of larval rearing.

138 H) Quantification of the number of NSC lineages non-individually encased from G).
139 Control (n = 8 VNCs) and *nrg RNAi* (n = 10 VNCs). Data statistics: Mann-Whitney test.
140 Results are presented as box and whisker plots.

141

142 **Figure 5. Individual encasing relies on balancing a CG to lineage interaction**
143 **through Nr_x-IV and Wrapper with an intra-lineage adhesion through Nrg.**

144 A) Representative confocal picture of the localisation of Wrapper in a thoracic VNC.
145 Larvae were dissected at ALH72 at 25°C. CG membrane is visualized by *Nrv2::GFP*
146 (green) and Wrapper is detected by a specific antibody (magenta).

147 B) Representative confocal picture of the thoracic VNC for a condition in which *wrapper*
148 is knocked down by RNAi in the CG (*CG > wrapper RNAi*, driver line *Nrv2::GFP*, *tub-*
149 *GAL80^{ts}*; *cyp4g15-GAL4*). Larvae are dissected after 68 h at 29°C from ALH0. CG
150 membrane is visualized by *Nrv2::GFP* (green) and NSCs are labelled with Dpn (grey).

151 C) Representative confocal picture of the thoracic VNC for a condition in which *wrapper*
152 is overexpressed in NSC lineages from ALH0 (*L^{NSC} > wrapper*, driver line *Nrv2::GFP*,
153 *wor-GAL4*; *tub-GAL80^{ts}*). Larvae are dissected after 68 h at 29°C. CG membrane is
154 visualized by *Nrv2::GFP* (green) and NSCs are labelled with Dpn (grey).

155 D) Schematic depicting the two isoforms for Nrg, Nrg¹⁶⁷ and Nrg¹⁸⁰. Only the
156 intracellular C-terminal part differs.

157 E) Representative confocal picture of the localisation of the Nrg¹⁸⁰ isoform in a thoracic
158 VNC, at ALH72 at 25°C. All Nrg isoforms are monitored through a *Nrg::GFP* protein
159 trap (green) and the Nrg¹⁸⁰ isoform is detected with a specific antibody (BP104, blue).

160 F) Representative confocal close-up picture of the respective localisations of the Nrg¹⁶⁷
161 and Nrg¹⁸⁰ isoform in a thoracic VNC, at ALH72 at 25°C. The Nrg¹⁶⁷ isoform is
162 visualized by a protein trap in the *nrg* gene leading to the preferential expression of
163 this isoform (*Nrg¹⁶⁷::GFP*, yellow). The Nrg¹⁸⁰ isoform is detected with a specific
164 antibody (BP104, blue). The dashed white line highlights the perimeter of the NSC
165 devoided of BP104 signal.

166 G) Representative confocal picture of a thoracic VNC and close-up for Nrg¹⁸⁰
167 overexpression in the CG from ALH0 (*CG > nrg¹⁸⁰*, driver *Nrv2::GFP*, *tub-GAL80^{ts}*;
168 *cyp4g15-GAL4*). Larvae are dissected after 68 h at 29°C. CG membrane is visualized

169 by *Nrv2::GFP* (green), NSCs are labelled with Dpn (grey) and *Nrg*¹⁸⁰ is detected with
170 a specific antibody (BP104, magenta).

171 H) Representative confocal picture of a thoracic VNC for *Nrg*¹⁶⁷ overexpression in the
172 CG from ALH0 (*CG > nrg*¹⁶⁷, driver *Nrv2::GFP*, *tub-GAL80^{ts}*; *cyp4g15-GAL4*). Larvae
173 are dissected after 68 h at 29°C. CG membrane is visualized by *Nrv2::GFP* (green)
174 and NSCs are labelled with Dpn (grey).

175 I) Representative confocal picture of a thoracic VNC for a condition in which *nrg*¹⁶⁷ is
176 overexpressed from ALH0 in NSC lineages (*L^{NSC} > nrg*¹⁶⁷, driver line *Nrv2::GFP*, *wor-*
177 *GAL4*; *tub-GAL80^{ts}*). Larvae are dissected after 68 h at 29°C. CG membrane is
178 visualized by *Nrv2::GFP* (green) and NSCs are labelled with Dpn (grey).

179 J) Quantification of the number of NSCs non-individually encased from I). Control
180 (*n* = 7 VNCs) and *nrg*¹⁶⁷ (*n* = 6 VNCs). Data statistics: Mann-Whitney test. Results are
181 presented as box and whisker plots.

182

183 **Figure 6. Loss of *Nrx-IV* and *Nrg* adhesions in NSC lineages during development**
184 **induces axonal misprojection from newborn neurons**

185 A) Schematic of the axonal projections coming from secondary, newborn neurons
186 generated by NSCs during larval neurogenesis. Only the VNC region is depicted.
187 1, antero-posterior view. 2, longitudinal view.

188 B) 3D reconstruction of a group of NSC lineages visualized with a membrane marker
189 (mTFP1-CAAX) in antero-posterior (1) and longitudinal (2) views for a control condition
190 and for *nrx-IV* knockdown in NSC lineages (*L^{NSC} > nrx-IV RNAi*). Clonal labelling was
191 obtained through the induction of Raeppli-CAAX in NSC lineages at ALH0. See
192 Methods for timing, conditions and genetics of larval rearing.

193 C) 3D reconstruction of a group of NSC lineages visualized with a membrane marker
194 (mTFP1-CAAX) in antero-posterior (1) and longitudinal (2) views for a control condition
195 and *nrg* knockdown in the NSC lineages (*L^{NSC} > nrg RNAi*). Clonal labelling was
196 obtained through the induction of Raeppli-CAAX in NSC lineages at ALH0. See
197 Methods for timing, conditions and genetics of larval rearing.

198 D) Schematic of the angle between the main axonal tract projecting from secondary
199 newborn neuron and the antero-posterior axis.

200 E) Quantification of the angle α_L depicted in D) in VNCs for control and *nrx-IV*
201 knockdown in NSC lineages, in the same conditions shown in B). Control (*n* = 149
202 axonal projections from 7 VNCs) and *nrx-IV RNAi* (*n* = 143 axonal projections from 8

203 VNCs). Data statistics: Mann-Whitney test. Results are presented as individual values,
204 the line represents the median.

205 F) Quantification of the angle α_L depicted in D) in VNCs for control and *nrg* knockdown
206 in NSC lineages, in the same conditions shown in C). Control (n = 144 axonal
207 projections from 7 VNCs) and *nrg* RNAi (n = 129 axonal projections from 6 VNCs).
208 Data statistics: Mann-Whitney test. Results are presented as individual values, the line
209 represents the median.

210

211 **Figure 7. Loss of NrX-IV and Nrg adhesions in NSC lineages during development**
212 **results in locomotor hyperactivity in the resulting adults**

213 A) Plot representing the percentage of global time sleeping (% asleep, ratio between
214 total sleep time and total time), in non-induced (flies always kept at 18°C before the
215 recordings) and induced (flies shifted to 29°C from early larval stage to mid-pupal
216 stage) conditions. Control, (*wor-GAL4, tub-Gal80^{ts} x w¹¹¹⁸*), n = 35 non-induced adult
217 males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4, tub-Gal80^{ts} x shg*
218 *RNAi^{VDR27082}*), n = 35 non-induced adult males and n = 55 induced adult males. *nrx-*
219 *IV* RNAi (*wor-GAL4, tub-Gal80^{ts} x nrx-IV RNAi^{BL32424}*), n = 35 non-induced adult males
220 and n = 55 induced adult males. *nrg* RNAi (*wor-GAL4, tub-Gal80^{ts} x nrg RNAi^{BL37496}*),
221 n = 35 non-induced adult males and n = 55 induced adult males.

222 B) Fraction (%) of the time sleeping across Light/Dark cycles (measured as the fraction
223 of time sleep within 30 min intervals) in non-induced (flies always kept at 18°C before
224 the recordings) and induced (flies shifted to 29°C from early larval stage to mid-pupal
225 stage) conditions. Control, (*wor-GAL4, tub-Gal80^{ts} x w¹¹¹⁸*), n = 35 non-induced adult
226 males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4, tub-Gal80^{ts} x shg*
227 *RNAi^{VDR27082}*), n = 35 non-induced adult males and n = 55 induced adult males. *nrx-*
228 *IV* RNAi (*wor-GAL4, tub-Gal80^{ts} x nrx-IV RNAi^{BL32424}*), n = 35 non-induced adult males
229 and n = 55 induced adult males. *nrg* RNAi (*wor-GAL4, tub-Gal80^{ts} x nrg RNAi^{BL37496}*),
230 n = 35 non-induced adult males and n = 55 induced adult males. Data statistics: one-
231 way ANOVA with a Kruskal–Wallis multiple comparison test. Results are presented as
232 box and whisker plots.

233 C) Mean velocity (in relative units) across Light/Dark cycles in non-induced (flies
234 always kept at 18°C before the recordings) and induced (flies shifted to 29°C from early
235 larval stage to mid-pupal stage) conditions. Control, (*wor-GAL4, tub-Gal80^{ts} x w¹¹¹⁸*),
236 n = 35 non-induced adult males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4,*

237 *tub-Gal80^{ts} x shg RNAi^{VDRG27082}*, n = 35 non-induced adult males and n = 55 induced
238 adult males. *nrx-IV RNAi (wor-GAL4, tub-Gal80^{ts} x nrx-IV RNAi^{BL32424})*, n = 35 non-
239 induced adult males and n = 55 induced adult males. *nrg RNAi (wor-GAL4, tub-Gal80^{ts}*
240 *x nrg RNAi^{BL37496})*, n = 35 non-induced adult males and n = 55 induced adult males.
241 Data statistics: one-way ANOVA with a Kruskal–Wallis multiple comparison test.
242 Results are presented as box and whisker plots.
243 D) Schematic depicting the timing and localisation of different adhesion complexes
244 within the NSC niche which are required for the individual encapsulation of NSC
245 lineages by CG. While *Nrx-IX* starts to be expressed in NSCs before encapsulation,
246 *Nrg* only appears afterwards, a timing preventing the clustering of NSCs, and hence
247 later on, NSC lineages, within one chamber. *Nrg* binds to itself in the NSC lineages
248 (dark blue complexes), while *Nrx-IV* binds to *Wrapper* expressed in the CG (pink
249 complexes). Adherens junctions (orange complexes) are also present between the
250 cells of the same NSC lineage, where they are mostly dispensible for individual
251 encasing, while potentially providing robustness.













