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

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2   **individual neural stem cell niches and controls adult behaviour**
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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

INTRODUCTION

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

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

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

3

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.

4

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 stereotypicencasing 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 dispensible for indvidual encasing. Further, we
83 demonstrated that the loss of Neurexin-IV and Neurglian 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

6

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.

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 on 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

7

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 ß-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 Raeppli-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   Raeppli-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

10

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.

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

11

198   immunoglobulin domains. Cont, homologous to the human Contactin, also contains

199   immunoglobulin domains, is GPI-anchored and only performs heterophilic interactions.

200   Nrx-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 (ATPα).

208   Interestingly, Nrx-IV and Nrg also perform nervous system-specific roles outside of the

209   septate junction. Nrx-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 Nrx-IV, Nrg, Cora, Dlg1 and ATPα are

217   enriched in NSC lineages.

218   First, using a protein trap (*Nrx-IV::GFP*, Fig. 4A), we found that Nrx-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), Nrx-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), Nrx-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, ATPα 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.

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 *ATPα* in NSC lineages using specific RNAi lines. Larvae from *dlg1*, *cont* and *ATPα*

244 knockdown died at early larval stages. From ATPα 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

13

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 Nrx-IV and Nrg in NSC lineages

278 are both required for their individual encasing by CG.

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

281 The dual role of Nrx-IV within and outside septate junctions is sustained by the

282 existence of alternative splicing [46]. Nrx-IV can be produced as a SJ isoform (Nrx-

283 IV^{exon3}), and a neuronal isoform outside of SJ (Nrx-IV^{exon4}). Nrx-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 Nrx-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

14

296   the highly characteristic pattern of large chambers (Fig. 5B) found during Nrx-IV

297   knockdown in NSC lineages (compare with Fig. 4C). Taken together, our results

298   suggest that Nrx-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 Nrx-IV to Wrapper interaction would fit in the hypothesis

303   of a difference in adhesion between A_L and $A_{L\text{-}CG}$. If CG to NSC lineage adhesion is

304   indeed weaker than intra-lineage adhesion (Fig. 1H, panel II.2), shifting Nrx-IV binding

305   to Wrapper from a glial to a NSC lineage pool (thus providing intra-lineage adhesion

306   through Nrx-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 $A_{L\text{-}CG}$.

308   However, if Nrx-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   ($A_L \approx A_{L\text{-}CG}$), and thus lead to random gouping 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 Nrx-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 Nrx-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, Nrg[167],

319   localizes in the SJ of epithelial tissues, the long isoform, Nrg[180], 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 Nrg[180] isoform

323   [56]. Staining of *Nrg::GFP* CNS (ALH72) with BP104 revealed that Nrg[180] 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

15

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

16

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 Nrx-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, $A_{NSC-NSC}$ 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 Nrg[167] (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 $A_{NSC-NSC}$ 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 **Nrx-IV and Nrg adhesions are required in NSC lineages for correct axonal path**
383 **during development**

384 So far, our data show that Nrx-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 *nrx-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 Nrx-IV adhesion in the niche are not critical for the
395 rate of NSC proliferation

17

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

18

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

19

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.

DISCUSSION

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

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

21

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, Nrx-IV-based interaction between CG and NSC lineages (A_{L-CG}

47 $< A_L$) builds the stereotyped, individual encasing of NSC lineages. There is no direct

48 mesure of the strength of adhesion between Nrx-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 Nrx-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-CG} 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 outweights the

56 presence of *de novo* adherens junctions between lineages and CG.

57 Nrx-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 Nrx-IV and

64 Wrapper interaction, CG still recognize NSC lineages as "wholes", but cannot

65 implement their individual encapsulation.

22

66   We propose that Nrg interact with itself in NSC lineages. We found that the Nrg[180]
67   isoform was expressed in newborn secondary neuron, in accordance with its known
68   neuronal association in other life stages. However, we propose that Nrg[167], 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 Nrg[167] isoform. We also noticed that
71   driving *nrg* RNAi in NSC lineages, under the same condition, completely abolished the
72   signal from Nrg[180] (BP104, Supp. Fig. 5C), but not from the *Nrg::GFP* fusion (Supp.
73   Fig. 4D. Whether Nrg[167] interacts with Nrg[180] for NSC encasing, or has another
74   junctional role, remains to be demonstrated. Of note, an elegant study has shown that
75   neuronal Nrg[180] can bind to epidermal Nrg[167] to prevent homologous Nrg[180]-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.

23

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
w[1118]	BDSC	5905
Nervana2::GFP (Nrv2::GFP)	BDSC	6828
Shg::GFP	Yohanns Bellaïche lab	
Nrx-IV::GFP	Christian Klämbt lab	*Nrx[454]*, [67]
Nrg::GFP	Kyoto	110658
Nrg[167]::GFP	BDSC	6844
Dlg1::GFP	BDSC	50859
ATPalpha::GFP (CPTI)	Kyoto DGGR	115323
tubulin-GAL80[thermosensitive(ts)]	BDSC	65406
yw, hs-FLP	Andrea Brand lab	
FRT G13	BDSC	1956
CoinFLP	BDSC	58750
cyp4g15-GAL4	BDSC	39103
cyp4g15-FRT-STOP-FRT-GAL4	This study	
cyp4g15-FRT-STOP-FRT-LexA	Spéder lab	[25]
cyp4g15-FLP	Spéder lab	[25]
cyp4g15-mtd::Tomato	Spéder lab	[25]
wor-Gal4	BDSC (Doe lab insertions)	56553 & 56554
PntP1-GAL4	Jan lab	[68]
CRIMIC wrapper	BDSC	93483
cyp4g15-QF2	Spéder lab	
tub-QS	BDSC	52112
TUG G13 MARCM line *y,w, hs-FLP; FRTG13, tubP-GAL80[LL2]/* *(CyO, act-GFP[JMR1]); tubP-GAL4[LL7],* *UAS-mCD8-GFP[LL6]/TM6B*	Bruno Bello	
tubP-GAL80[LL2]	BDSC	5140
UAS-reaper	Andrea Brand lab	
UAS-mCD8::GFP	BDSC	5130
UAS-mCD8::RFP	BDSC	27399
UAS-Raeppli CAAX 43E	Generated from BDSC 55082	[25]
UAS-Raeppli NLS 53D	Generated from BDSC 55087	[25]
UAS-Raeppli NLS 89A	Generated from BDSC 55088	[25]
UAS-prospero RNAi	Andrea Brand lab	
UAS-brat RNAi	Andrea Brand lab	

25

UAS-aPKc		[69]
UAS-shg RNAi	BDSC	32904
	VDRC	27082
	VDRC	103962
UAS-arm RNAi	VDRC	27227
UAS-nrx IV RNAi	BDSC	32424
UAS-nrg RNAi	BDSC	37496
UAS-wrapper RNAi	BDSC	29561
UAS-ATPα RNAI	BDSC	28073
UAS-nrg^{180}	BDSC	24169
UAS-nrg^{167}	BDSC	24172
UAS-nrg^{GPI}	BDSC	24168
UAS-wrapper	BDSC	78535
QUAS-PTEN	This study	
shg^{null} (shg^{R64a})	Yohanns Bellaïche lab	

135

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 quinin acid (dissolved in sterile water) into melted food at 50°C, for a final

144 concentration of 20 mg/ml of quinic acid.

145

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

26

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 side (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-Nrg[180] (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-

27

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   haching (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).

29

Clonal analyses using CoinFLP

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

Quantification of NSC lineage encasing (Raeppli-NLS)

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

Quantification of NSC mitotic index

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

Quantification of axonal angle

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

Behavioural analysis

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

30

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.

Acknowledgments

Author Contributions

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

Declaration of Interests

The authors declare no competing interest.

Data availability statement

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

References

1. Ferraro, F., Lo Celso, C., and Scadden, D. (2010). Adult stem cells and their niches. Adv. Exp. Med. Biol. *695*, 155–168. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21222205 [Accessed May 12, 2020].

2. Lander, A.D., Kimble, J., Clevers, H., Fuchs, E., Montarras, D., Buckingham, M., Calof, A.L., Trumpp, A., and Oskarsson, T. (2012). What does the concept of the stem cell niche really mean today? BMC Biol. *10*, 19. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3298504&tool=pmcentrez&rendertype=abstract [Accessed April 3, 2016].

3. Chacón-Martínez, C.A., Koester, J., and Wickström, S.A. (2018). Signaling in the stem cell niche: regulating cell fate, function and plasticity. Development *145*, dev165399.

4. Decimo, I., Bifari, F., Krampera, M., and Fumagalli, G. (2012). Neural stem cell niches in health and diseases. Curr. Pharm. Des. *18*, 1755–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/22394166 [Accessed October 14, 2019].

5. Obernier, K., and Alvarez-Buylla, A. (2019). Neural stem cells: Origin, heterogeneity and regulation in the adult mammalian brain. Dev. *146*.

6. Randolph S. Ashton, Anthony Conway, Chinmay Pangarkar, J.B., and Kwang-Il Lim, Priya Shah, Mina Bissell, and D.V.S. (2012). Astrocytes regulate adult hippocampal neurogenesis through ephrin-B signaling. Nat. Neurosci. *9*, 47–55.

7. Paul, A., Chaker, Z., and Doetsch, F. (2017). Hypothalamic regulation of regionally distinct adult neural stem cells and neurogenesis. Science (80-.). *356*, 1383–1386. Available at: http://www.sciencemag.org/lookup/doi/10.1126/science.aal3839.

8. Chell, J.M., and Brand, A.H. (2010). Nutrition-responsive glia control exit of neural stem cells from quiescence. Cell *143*, 1161–73. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3087489&tool=pmcentrez&rendertype=abstract [Accessed February 27, 2013].

9. Sousa-Nunes, R., Yee, L.L., and Gould, A.P. (2011). Fat cells reactivate quiescent neuroblasts via TOR and glial insulin relays in Drosophila. Nature *471*, 508–12. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3146047&tool=pmcentrez&rendertype=abstract [Accessed February 27, 2013].

10. Homem, C.C.F., Repic, M., and Knoblich, J.A. (2015). Proliferation control in neural stem and progenitor cells. Nat. Rev. Neurosci. *16*, 647–59. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26420377 [Accessed April 12, 2016].

11. Bello, B.C., Izergina, N., Caussinus, E., and Reichert, H. (2008). Amplification of neural stem cell proliferation by intermediate progenitor cells in Drosophila brain development. Neural Dev. *3*, 5. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2265709&tool=pmcentrez&rendertype=abstract [Accessed March 1, 2013].

12. Boone, J.Q., and Doe, C.Q. (2008). Identification of Drosophila type II neuroblast lineages containing transit amplifying ganglion mother cells. Dev. Neurobiol. *68*, 1185–95. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2804867&tool=pmcentrez&rendertype=abstract [Accessed March 4, 2013].

13. Bowman, S.K., Rolland, V., Betschinger, J., Kinsey, K. a, Emery, G., and Knoblich, J. a (2008). The tumor suppressors Brat and Numb regulate transit-amplifying neuroblast lineages in Drosophila. Dev. Cell *14*, 535–46. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2988195&tool=pmcentrez&rendertype=abstract [Accessed March 1, 2013].

14. Spéder, P., and Brand, A.H. (2014). Gap Junction Proteins in the Blood-Brain Barrier Control Nutrient-Dependent Reactivation of Drosophila Neural Stem Cells. Dev. Cell, 309–321. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25065772 [Accessed August 1, 2014].

15. Kanai, M.I., Kim, M.-J., Akiyama, T., Takemura, M., Wharton, K., O'Connor, M.B., and Nakato, H. (2018). Regulation of neuroblast proliferation by surface glia in the Drosophila larval brain. Sci. Rep. *8*, 3730. Available at: http://www.nature.com/articles/s41598-018-22028-y [Accessed May 24, 2019].

16. Morante, J., Vallejo, D.M., Desplan, C., and Dominguez, M. (2013). Conserved miR-8/miR-200 defines a glial niche that controls neuroepithelial expansion and neuroblast transition. Dev. Cell *27*, 174–187. Available at: https://pubmed.ncbi.nlm.nih.gov/24139822/ [Accessed August 5, 2022].

17. Plazaola-Sasieta, H., Zhu, Q., Gaitán-Peñas, H., Rios, M., Estévez, R., and Morey, M. (2019). Drosophila ClC-a is required in glia of the stem cell niche for proper neurogenesis and wiring of neural circuits. Glia *67*, 2374–2398. Available at: https://onlinelibrary.wiley.com/doi/full/10.1002/glia.23691 [Accessed April 7, 2021].

18. Dong, Q., Zavortink, M., Froldi, F., Golenkina, S., Lam, T., and Cheng, L.Y. (2021). Glial Hedgehog signalling and lipid metabolism regulate neural stem cell proliferation in Drosophila . EMBO Rep. Available at: https://pubmed.ncbi.nlm.nih.gov/33751817/ [Accessed April 18, 2021].

19. Cheng, L.Y., Bailey, A.P., Leevers, S.J., Ragan, T.J., Driscoll, P.C., and Gould, A.P. (2011). Anaplastic lymphoma kinase spares organ growth during nutrient restriction in Drosophila. Cell *146*, 435–47. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21816278 [Accessed February 27, 2013].

20. Bailey, A.P., Koster, G., Guillermier, C., Hirst, E.M.A., MacRae, J.I., Lechene, C.P., Postle, A.D., and Gould, A.P. (2015). Antioxidant Role for Lipid Droplets in a Stem Cell Niche of Drosophila. Cell *163*, 340–353. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4601084&tool=pmcentrez&rendertype=abstract [Accessed October 9, 2015].

21. Spéder, P., and Brand, A.H. (2018). Systemic and local cues drive neural stem cell niche remodelling during neurogenesis in Drosophila. Elife *7*, e30413. Available at: https://elifesciences.org/articles/30413 [Accessed July 8, 2018].

22. Dumstrei, K., Wang, F., and Hartenstein, V. (2003). Role of DE-cadherin in neuroblast proliferation, neural morphogenesis, and axon tract formation in Drosophila larval brain development. J. Neurosci. *23*, 3325–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12716940.

23. Pereanu, W., Shy, D., and Hartenstein, V. (2005). Morphogenesis and proliferation of the larval brain glia in Drosophila. Dev. Biol. *283*, 191–203. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15907832 [Accessed March 1, 2013].

24. Yuan, X., Sipe, C.W., Suzawa, M., Bland, M.L., and Siegrist, S.E. (2020). Dilp-2-mediated PI3-kinase activation coordinates reactivation of quiescent neuroblasts with

91 growth of their glial stem cell niche. PLoS Biol. *18*, 1–24. Available at:
92 http://dx.doi.org/10.1371/journal.pbio.3000721.

93 25. Rujano, M.A., Briand, D., Đelić, B., Marc, J., and Spéder, P. (2022). An interplay
94 between cellular growth and atypical fusion defines morphogenesis of a modular glial
95 niche in Drosophila. Nat. Commun. *13*. Available at:
96 https://pubmed.ncbi.nlm.nih.gov/36008397/ [Accessed September 26, 2022].

97 26. Spindler, S.R., and Hartenstein, V. (2010). The Drosophila neural lineages: a model
98 system to study brain development and circuitry. Dev. Genes Evol. *220*, 1–10.
99 Available at: http://www.ncbi.nlm.nih.gov/pubmed/20306203 [Accessed June 21,
100 2018].

101 27. Larsen, C., Shy, D., Spindler, S.R., Fung, S., Pereanu, W., Younossi-Hartenstein, A.,
102 and Hartenstein, V. (2009). Patterns of growth, axonal extension and axonal
103 arborization of neuronal lineages in the developing Drosophila brain. Dev. Biol. *335*,
104 289. Available at: /pmc/articles/PMC2785225/ [Accessed September 29, 2022].

105 28. Spindler, S.R., Ortiz, I., Fung, S., Takashima, S., and Hartenstein, V. (2009).
106 Drosophila cortex and neuropile glia influence secondary axon tract growth,
107 pathfinding, and fasciculation in the developing larval brain. Dev. Biol. *334*, 355–368.

108 29. Freeman, M.R. (2015). Drosophila Central Nervous System Glia. Cold Spring Harb.
109 Perspect. Biol. *7*. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25722465
110 [Accessed June 14, 2018].

111 30. Kang, K.H., and Reichert, H. (2015). Control of neural stem cell self-renewal and
112 differentiation in Drosophila. Cell Tissue Res. *359*, 33–45. Available at:
113 http://www.ncbi.nlm.nih.gov/pubmed/24902665 [Accessed October 30, 2015].

114 31. Choksi, S.P., Southall, T.D., Bossing, T., Edoff, K., de Wit, E., Fischer, B.E., van
115 Steensel, B., Micklem, G., and Brand, A.H. (2006). Prospero acts as a binary switch
116 between self-renewal and differentiation in Drosophila neural stem cells. Dev Cell *11*,
117 775–789. Available at:
118 http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citati
119 on&list_uids=17141154.

120 32. Bello, B., Reichert, H., and Hirth, F. (2006). The brain tumor gene negatively regulates
121 neural progenitor cell proliferation in the larval central brain of Drosophila.
122 Development *133*, 2639–2648.

123 33. Betschinger, J., Mechtler, K., and Knoblich, J. a (2006). Asymmetric segregation of the
124 tumor suppressor brat regulates self-renewal in Drosophila neural stem cells. Cell *124*,
125 1241–1253. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16564014 [Accessed
126 February 27, 2013].

127 34. Kanca, O., Caussinus, E., Denes, A.S., Percival-Smith, A., and Affolter, M. (2014).
128 Raeppli: a whole-tissue labeling tool for live imaging of Drosophila development.
129 Development *141*, 472–480. Available at:
130 http://www.ncbi.nlm.nih.gov/pubmed/24335257.

131 35. Foty, R.A., and Steinberg, M.S. (2013). Differential adhesion in model systems. Wiley
132 Interdiscip. Rev. Dev. Biol. *2*, 631–645. Available at:
133 https://onlinelibrary.wiley.com/doi/full/10.1002/wdev.104 [Accessed September 5,
134 2022].

135 36. Bosch, J.A., Tran, N.H., and Hariharan, I.K. (2015). CoinFLP: a system for efficient

136   mosaic screening and for visualizing clonal boundaries in Drosophila. Development
137   *142*, 597–606. Available at: http://dev.biologists.org/cgi/doi/10.1242/dev.114603.

138 37. White, K., Grether, M.E., Abrams, J.M., Young, L., Farrell, K., and Steller, H. (1994).
139   Genetic control of programmed cell death in Drosophila. Science (80-.). *264*, 677–
140   683. Available at:
141   http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citati
142   on&list_uids=8171319.

143 38. Coutinho-Budd, J.C., Sheehan, A.E., and Freeman, M.R. (2017). The secreted
144   neurotrophin spätzle 3 promotes glial morphogenesis and supports neuronal survival
145   and function. Genes Dev. *31*, 2023–2038.

146 39. Almeida, M.S., and Bray, S.J. (2005). Regulation of post-embryonic neuroblasts by
147   Drosophila Grainyhead. Mech. Dev. *122*, 1282–1293.

148 40. Nériec, N., and Desplan, C. (2016). From the Eye to the Brain: Development of the
149   Drosophila Visual System. Curr. Top. Dev. Biol. *116*, 247–71. Available at:
150   http://www.ncbi.nlm.nih.gov/pubmed/26970623 [Accessed April 4, 2016].

151 41. Doyle, S.E., Pahl, M.C., Siller, K.H., Ardiff, L., and Siegrist, S.E. (2017). Neuroblast
152   niche position is controlled by phosphoinositide 3-kinase-dependent DE-cadherin
153   adhesion. Dev. *144*, 820–829.

154 42. Hortsch, M., and Margolis, B. (2003). Septate and paranodal junctions: kissing
155   cousins. Trends Cell Biol. *13*, 557–561.

156 43. Zihni, C., Mills, C., Matter, K., and Balda, M.S. (2016). Tight junctions: from simple
157   barriers to multifunctional molecular gates. Nat. Rev. Mol. Cell Biol. 2016 179 *17*, 564–
158   580. Available at: https://www.nature.com/articles/nrm.2016.80 [Accessed September
159   30, 2022].

160 44. Baumgartner, S., Littleton, J.T., Broadie, K., Bhat, M. a, Harbecke, R., Lengyel, J. a,
161   Chiquet-Ehrismann, R., Prokop, a, and Bellen, H.J. (1996). A Drosophila neurexin is
162   required for septate junction and blood-nerve barrier formation and function. Cell *87*,
163   1059–68. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8978610.

164 45. Noordermeer, J.N., Kopczynski, C.C., Fetter, R.D., Bland, K.S., Chen, W.Y., and
165   Goodman, C.S. (1998). Wrapper, a novel member of the Ig superfamily, is expressed
166   by midline glia and is required for them to ensheath commissural axons in Drosophila.
167   Neuron *21*, 991–1001. Available at: https://pubmed.ncbi.nlm.nih.gov/9856456/
168   [Accessed August 29, 2022].

169 46. Stork, T., Thomas, S., Rodrigues, F., Silies, M., Naffin, E., Wenderdel, S., and Klämbt,
170   C. (2009). Drosophila Neurexin IV stabilizes neuron-glia interactions at the CNS
171   midline by binding to Wrapper. Development *136*, 1251–61. Available at:
172   http://www.ncbi.nlm.nih.gov/pubmed/19261699 [Accessed March 4, 2013].

173 47. Wheeler, S.R., Banerjee, S., Blauth, K., Rogers, S.L., Bhat, M.A., and Crews, S.T.
174   (2009). Neurexin IV and Wrapper interactions mediate Drosophila midline glial
175   migration and axonal ensheathment. Development *136*, 1147–1157. Available at:
176   https://journals.biologists.com/dev/article/136/7/1147/65448/Neurexin-IV-and-
177   Wrapper-interactions-mediate [Accessed September 6, 2022].

178 48. Yamamoto, M., Ueda, R., Takahashi, K., Saigo, K., and Uemura, T. (2006). Control of
179   Axonal Sprouting and Dendrite Branching by the Nrg-Ank Complex at the Neuron-Glia
180   Interface. Curr. Biol. *16*, 1678–1683.

181   49.   Martin, V., Mrkusich, E., Steinel, M.C., Rice, J., Merritt, D.J., and Whitington, P.M.
182   (2008). The L1-type cell adhesion molecule Neuroglian is necessary for maintenance
183   of sensory axon advance in the Drosophila embryo. Neural Dev. *3*, 1–11. Available at:
184   https://neuraldevelopment.biomedcentral.com/articles/10.1186/1749-8104-3-10
185   [Accessed September 6, 2022].

186   50.   Yang, W.K., Chueh, Y.R., Cheng, Y.J., Siegenthaler, D., Pielage, J., and Chien, C.T.
187   (2019). Epidermis-Derived L1CAM Homolog Neuroglian Mediates Dendrite Enclosure
188   and Blocks Heteroneuronal Dendrite Bundling. Curr. Biol. *29*, 1445-1459.e3.

189   51.   Goossens, T., Kang, Y.Y., Wuytens, G., Zimmermann, P., Callaerts-Végh, Z.,
190   Pollarolo, G., Islam, R., Hortsch, M., and Callaerts, P. (2011). The Drosophila L1CAM
191   homolog Neuroglian signals through distinct pathways to control different aspects of
192   mushroom body axon development. Development *138*, 1595–1605. Available at:
193   https://pubmed.ncbi.nlm.nih.gov/21389050/ [Accessed September 9, 2022].

194   52.   Clements, J., Buhler, K., Winant, M., Vulsteke, V., and Callaerts, P. (2021). Glial and
195   Neuronal Neuroglian, Semaphorin-1a and Plexin A Regulate Morphological and
196   Functional Differentiation of Drosophila Insulin-Producing Cells. Front. Endocrinol.
197   (Lausanne). *12*. Available at: https://pubmed.ncbi.nlm.nih.gov/34276554/ [Accessed
198   September 30, 2022].

199   53.   Shepherd, D., Harris, R., Williams, D.W., and Truman, J.W. (2016). Postembryonic
200   lineages of the Drosophila ventral nervous system: Neuroglian expression reveals the
201   adult hemilineage associated fiber tracts in the adult thoracic neuromeres. J. Comp.
202   Neurol. *524*, 2677–2695. Available at:
203   https://onlinelibrary.wiley.com/doi/full/10.1002/cne.23988 [Accessed September 9,
204   2022].

205   54.   Nakano, R., Iwamura, M., Obikawa, A., Togane, Y., Hara, Y., Fukuhara, T., Tomaru,
206   M., Takano-Shimizu, T., and Tsujimura, H. (2019). Cortex glia clear dead young
207   neurons via Drpr/dCed-6/Shark and Crk/Mbc/dCed-12 signaling pathways in the
208   developing Drosophila optic lobe. Dev. Biol. Available at:
209   https://www.sciencedirect.com/science/article/pii/S0012160618305645?via%3Dihub
210   [Accessed May 13, 2019].

211   55.   Lee, P.T., Zirin, J., Kanca, O., Lin, W.W., Schulze, K.L., Li-Kroeger, D., Tao, R.,
212   Devereaux, C., Hu, Y., Chung, V., *et al.* (2018). A gene-specific T2A-GAL4 library for
213   drosophila. Elife *7*.

214   56.   Hortsch, M., Bieber, A.J., Patel, N.H., and Goodman, C.S. (1990). Differential splicing
215   generates a nervous system-specific form of Drosophila neuroglian. Neuron *4*, 697–
216   709. Available at: https://pubmed.ncbi.nlm.nih.gov/1693086/ [Accessed September 13,
217   2022].

218   57.   Hortsch, M., Bieber, A.J., Patel, N.H., and Goodman, C.S. (1990). Differential splicing
219   generates a nervous system—Specific form of drosophila neuroglian. Neuron *4*, 697–
220   709.

221   58.   Islam, R., Kristiansen, L. V., Romani, S., Garcia-Alonso, L., and Hortsch, M. (2004).
222   Activation of EGF receptor kinase by L1-mediated homophilic cell interactions. Mol.
223   Biol. Cell *15*, 2003–2012. Available at: https://pubmed.ncbi.nlm.nih.gov/14718570/
224   [Accessed September 20, 2022].

225   59.   Goossens, T., Kang, Y.Y., Wuytens, G., Zimmermann, P., Callaerts-Végh, Z.,
226   Pollarolo, G., Islam, R., Hortsch, M., and Callaerts, P. (2011). The Drosophila L1CAM
227   homolog Neuroglian signals through distinct pathways to control different aspects of

228   mushroom body axon development. Development *138*, 1595–1605. Available at:
229   https://pubmed.ncbi.nlm.nih.gov/21389050/ [Accessed September 6, 2022].

230 60. Geissmann, Q., Garcia Rodriguez, L., Beckwith, E.J., French, A.S., Jamasb, A.R., and
231   Gilestro, G.F. (2017). Ethoscopes: An open platform for high-throughput ethomics.
232   PLOS Biol. *15*, e2003026. Available at:
233   https://journals.plos.org/plosbiology/article?id=10.1371/journal.pbio.2003026
234   [Accessed September 15, 2022].

235 61. Beckwith, E.J., and French, A.S. (2019). Sleep in Drosophila and Its Context. Front.
236   Physiol. *10*, 1167. Available at: /pmc/articles/PMC6749028/ [Accessed February 22,
237   2022].

238 62. Kashima, R., Redmond, P.L., Ghatpande, P., Roy, S., Kornberg, T.B., Hanke, T.,
239   Knapp, S., Lagna, G., and Hata, A. (2017). Hyperactive locomotion in a Drosophila
240   model is a functional readout for the synaptic abnormalities underlying fragile X
241   syndrome. Sci. Signal. *10*. Available at:
242   https://www.science.org/doi/10.1126/scisignal.aai8133 [Accessed September 30,
243   2022].

244 63. Klein, M., Singgih, E.L., van Rens, A., Demontis, D., Børglum, A.D., Mota, N.R.,
245   Castells-Nobau, A., Kiemeney, L.A., Brunner, H.G., Arias-Vasquez, A., *et al.* (2020).
246   Contribution of Intellectual Disability-Related Genes to ADHD Risk and to Locomotor
247   Activity in Drosophila. Am. J. Psychiatry *177*, 526–536. Available at:
248   https://pubmed.ncbi.nlm.nih.gov/32046534/ [Accessed September 30, 2022].

249 64. Takai, A., Chiyonobu, T., Ueoka, I., Tanaka, R., Tozawa, T., Yoshida, H., Morimoto,
250   M., Hosoi, H., and Yamaguchi, M. (2020). A novel Drosophila model for
251   neurodevelopmental disorders associated with Shwachman–Diamond syndrome.
252   Neurosci. Lett. *739*, 135449.

253 65. Yang, Z., Yu, Y., Zhang, V., Tian, Y., Qi, W., and Wang, L. (2015). Octopamine
254   mediates starvation-induced hyperactivity in adult Drosophila. Proc. Natl. Acad. Sci. U.
255   S. A. *112*, 5219–5224. Available at:
256   https://www.pnas.org/doi/abs/10.1073/pnas.1417838112 [Accessed September 30,
257   2022].

258 66. Yu, Y., Huang, R., Ye, J., Zhang, V., Wu, C., Cheng, G., Jia, J., and Wang, L. (2016).
259   Regulation of starvation-induced hyperactivity by insulin and glucagon signaling in
260   adult Drosophila. Elife *5*.

261 67. Edenfeld, G., Volohonsky, G., Krukkert, K., Naffin, E., Lammel, U., Grimm, A.,
262   Engelen, D., Reuveny, A., Volk, T., Klambt, C., *et al.* (2006). The splicing factor
263   crooked neck associates with the RNA-binding protein HOW to control glial cell
264   maturation in Drosophila. Neuron *52*, 969–980. Available at:
265   http://www.ncbi.nlm.nih.gov/pubmed/17178401 [Accessed March 10, 2013].

266 68. Zhu, S., Barshow, S., Wildonger, J., Jan, L.Y., and Jan, Y.-N. (2011). Ets transcription
267   factor Pointed promotes the generation of intermediate neural progenitors in
268   Drosophila larval brains. Proc. Natl. Acad. Sci. U. S. A. *108*, 20615–20. Available at:
269   http://www.ncbi.nlm.nih.gov/pubmed/22143802 [Accessed October 4, 2022].

270 69. Sotillos, S., Díaz-Meco, M.T., Caminero, E., Moscat, J., and Campuzano, S. (2004).
271   DaPKC-dependent phosphorylation of Crumbs is required for epithelial cell polarity in
272   Drosophila. J. Cell Biol. *166*, 549–557. Available at:
273   http://www.jcb.org/cgi/doi/10.1083/jcb.200311031 [Accessed September 26, 2022].

274   70.   Pfeiffer, B.D., Jenett, A., Hammonds, A.S., Ngo, T.-T.B., Misra, S., Murphy, C., Scully,
275   A., Carlson, J.W., Wan, K.H., Laverty, T.R., *et al.* (2008). Tools for neuroanatomy and
276   neurogenetics in Drosophila. Proc. Natl. Acad. Sci. U. S. A. *105*, 9715–20. Available
277   at:
278   http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2447866&tool=pmcentrez&r
279   endertype=abstract.

280   71.   Petersen, L.K., and Stowers, R.S. (2011). A Gateway MultiSite recombination cloning
281   toolkit. PLoS One *6*, e24531. Available at:
282   http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3170369&tool=pmcentrez&r
283   endertype=abstract [Accessed March 9, 2016].

284   72.   Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., and Xu, T.
285   (1999). PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye
286   development. Development *126*, 5365–5372. Available at:
287   https://pubmed.ncbi.nlm.nih.gov/10556061/ [Accessed February 21, 2022].

288   73.   Benmimoun, B., Papastefanaki, F., Périchon, B., Segklia, K., Roby, N., Miriagou, V.,
289   Schmitt, C., Dramsi, S., Matsas, R., and Spéder, P. (2020). An original infection model
290   identifies host lipoprotein import as a route for blood-brain barrier crossing. Nat.
291   Commun. *11*, 1–18. Available at: https://doi.org/10.1038/s41467-020-19826-2
292   [Accessed March 9, 2021].

293   74.   Geissmann, Q., Rodriguez, L.G., Beckwith, E.J., and Gilestro, G.F. (2019). Rethomics:
294   An R framework to analyse high-throughput behavioural data. PLoS One *14*. Available
295   at: https://pubmed.ncbi.nlm.nih.gov/30650089/ [Accessed October 2, 2022].

296

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).

41

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).

100

42

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 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 Raeppli-NLS (blue, white, orange and red). Raeppli-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 Raeppli-NLS (blue, white, orange and red). Raeppli-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 Nrx-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, Nrg^{167} and Nrg^{180}. Only the

156   intracellular C-terminal part differs.

157   E) Representative confocal picture of the localisation of the Nrg^{180} 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 Nrg^{180} isoform is detected with a specific antibody (BP104, blue).

160   F) Representative confocal close-up picture of the respective localisations of the Nrg^{167}

161   and Nrg^{180} isoform in a thoracic VNC, at ALH72 at 25°C. The Nrg^{167} isoform is

162   visualized by a protein trap in the *nrg* gene leading to the preferential expression of

163   this isoform (*Nrg^{167}::GFP*, yellow). The Nrg^{180} 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 Nrg^{180}

167   overexpression in the CG from ALH0 (*CG > nrg^{180}*, driver *Nrv2::GFP, tub-GAL80^{ts};*

168   *cyp4g15-GAL4*). Larvae are dissected after 68 h at 29°C. CG membrane is visualized

44

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

45

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 w^{1118}*), n = 35 non-induced adult

217 males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4, tub-Gal80^{ts} x shg*

218 *RNAi^{VDRC27082}*), 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 w^{1118}*), n = 35 non-induced adult

226 males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4, tub-Gal80^{ts} x shg*

227 *RNAi^{VDRC27082}*), 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 w^{1118}*),

236 n = 35 non-induced adult males and n = 55 induced adult males. *shg* RNAi (*wor-GAL4,*

46

237   *tub-Gal80ᵗˢ x shg RNAiᵛᴰᴿᶜ²⁷⁰⁸²*), n = 35 non-induced adult males and n = 55 induced

238   adult males. *nrx-IV* RNAi (*wor-GAL4, tub-Gal80ᵗˢ x nrx-IV RNAIᴮᴸ³²⁴²⁴*), n = 35 non-

239   induced adult males and n = 55 induced adult males. *nrg* RNAi (*wor-GAL4, tub-Gal80ᵗˢ*

240   *x nrg RNAIᴮᴸ³⁷⁴⁹⁶*), 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.

A CB, OL, VNC

B Type I: NSC → GMC → neurons; Type II: NSC → imINP → mINP → GMC → neurons

C CB, VNC — GFP, NSC, Neuron, Glia nuclei — 20 µm

D Perineural glia, Subperineural glia, Cortex glia, Neuroblast, GMC/INP, Neurons

E CG: growth, chamber formation, extension; NSC: quiescence, reactivation, cycling; nutritional signal

F control, pros RNAi — CG membrane, NSC/tumour, Neuron — 5 µm; control, brat RNAi — CG membrane, NSC/tumour, Ase, Pros — 20 µm

G control, pros RNAi — CG membrane, NSC lineages — 5 µm, 10 µm; control, brat RNAi — CG membrane, NSC lineages — 20 µm

H

I. NSC driven mechanism — TIMING

II. Adhesion
  II.1. Specific pairing
  II.2. Differential adhesion mechanism

A (L-CG)
∧
A (L)

Banach-Latapy et al, Figure 2

A

PI3K/Akt OFF in CG ➡ no growth   PI3K/Akt ON in CG ➡ growth

ALH   0   24   48   72   96

T1   T2

B

Control   Delayed chamber (PTEN)

T2   20 µm   T2   20 µm

10 µm   10 µm

CG membrane NSC lineages

C

□ Control   □ Delayed chamber

NSC lineages non encased individually

0.0373

D

?

E

Control

PTEN clone

10 µm   10 µm

CG membrane NSC Clone membrane

A — CG membrane (green) NSC (white) Neuron (blue) Shg (magenta); panels ALH2, ALH24, ALH48, ALH72

B — ALH72; Arm (magenta)

C — Control; L^{NSC} > shg RNAi; CG membrane (green) NSC (white)

D — L^{NSC} > shg RNAi; CG membrane (green) NSC (white) C lineages (red/blue/orange)

E — shg RNAi; PI3K/Akt OFF in CG → no growth; PI3K/Akt ON in CG → growth; ALH 0 24 48 72 96; T1; T2

F — T2; CG membrane (green) NSC (white) C lineages

G — Control; Delayed chamber; Delayed chamber + shg knockdown; # NSC lineages non encased individually; 0.0047; 0.3776; 0.7538

H — CG> shg; CG membrane (green) Shg (magenta) NSC (white) Neuron (blue)

A — CG membrane NSC Neuron Nrx-IV::GFP

B — CG membrane Dpn ElaV Nrg::GFP

C — L^NSC > nrx-IV RNAi — CG membrane NSC

F — L^NSC > nrg RNAi — CG membrane NSC

D — L^NSC > nrx-IV RNAi

G — L^NSC > nrg RNAi

CG membrane NSC lineages

E — Control / L^NSC > nrx-IV RNAi — # NSC lineages non encased individually — <0.0001

H — Control / L^NSC > nrg RNAi — # NSC lineages non encased individually — 0.0001

A CG membrane Wrapper

ALH72

B *CG > wrapper RNAi*
CG membrane NSC

C *L^NSC > wrapper*
CG membrane NSC Wrapper

D Nrg^167 Nrg^180
Ig-like domains
Fn type III repeats

E ALH72
Nrg::GFP Nrg^180

F ALH72
Nrg^167::GFP Nrg^180

G *CG > nrg^180*
CG membrane NSC Nrg

H *CG > nrg^167*
CG membrane NSC

I *L^NSC > nrg^167*
CG membrane NSC

J
☐ Control
☐ *L^NSC > nrg^167*

NSCs non encased individually

0.0012

A

1 | 2

1

Antero-posterior view

2

Longitudinal view

D

A — P

α_L

B

Control | 1

| 2

20 µm

L^{NSC} > nrx-IV RNAi | 1

| 2

20 µm

E

α_L in °

< 0.0001

L^{NSC} > - nrx-IV RNAi

C

Control | 1

| 2

20 µm

L^{NSC} > nrg RNAi | 1

| 2

20 µm

F

α_L in °

0.0127

L^{NSC} > - nrg RNAi