L1CAM/Neuroglian controls the axon–axon interactions establishing layered and lobular mushroom body architecture

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The establishment of neuronal circuits depends on the guidance of axons both along and in between axonal populations of different identity; however, the molecular principles controlling axon–axon interactions in vivo remain largely elusive. We demonstrate that the Drosophila melanogaster L1CAM homologue Neuroglian mediates adhesion between functionally distinct mushroom body axon populations to enforce and control appropriate projections into distinct axonal layers and lobes essential for olfactory learning and memory. We addressed the regulatory mechanisms controlling homophilic Neuroglian-mediated cell adhesion by analyzing targeted mutations of extra- and intracellular Neuroglian domains in combination with cell type-specific rescue assays in vivo. We demonstrate independent and cooperative domain requirements: intercalating growth depends on homophilic adhesion mediated by extracellular Ig domains. For functional cluster formation, intracellular Ankyrin2 association is sufficient on one side of the trans-axonal complex whereas Moesin association is likely required simultaneously in both interacting axonal populations. Together, our results provide novel mechanistic insights into cell adhesion molecule-mediated axon–axon interactions that enable precise assembly of complex neuronal circuits.

Introduction

The ability of the brain to process and store information requires the assembly of neurons into complex circuits. This process depends on appropriate guidance of axons to distant targets. Although significant progress has been made regarding the identification of signaling systems controlling long-range axon guidance (Kolodkin and Tessier-Lavigne, 2011), the molecular and cellular mechanisms controlling axon–axon interactions between neuronal populations at guidance choice points in vivo remain largely unknown. To address the molecular mechanisms controlling the establishment of complex axonal assemblies, we used the Drosophila mushroom bodies (MBs), a bilaterally symmetric central brain structure essential for olfactory learning and memory, as a model system (de Belle and Heisenberg, 1994). The MBs are composed of ~2,000 Kenyon cells (KCs; Heisenberg, 2003) derived from four neuroblasts (NBs) that in a sequential manner give rise to three genetically, anatomically, and functionally distinct subpopulations (β, αβ', and αβ neurons; Crittenden et al., 1998; Lee et al., 1999; Krashes et al., 2007; Trannoy et al., 2011). During embryonic and early larval development, axons of γ neurons fasciculate into a single bundle below the MB calyx and project via the pedunculus, the major axonal MB tract, to the anterior brain, where they branch into medial and vertical lobes. During late larval stages, αβ' axons are born (Lee and Luo, 1999) that form a subtype-specific fascicle and intercalate at the center of the pedunculus in between γ axons (Kurusu et al., 2002). At the end of the pedunculus (pedunculus divide), the axons branch to form medial and vertical lobes in close proximity to γ lobes. Finally, at early pupal stages, axons of αβ neurons intercalate in between αβ' neurons, thereby forming a third concentric axonal layer within the pedunculus (Kurusu et al., 2002) and vertical and medial lobes next to αβ' lobes in the anterior brain (Fig. 1 A). Thus, MB axons of different identity form highly associated but strictly segregated axonal layers and lobes. The importance of the anatomical segregation is reflected...
Figure 1. **Extra- and intracellular Nrg domains contribute to MB axon guidance.** [A] Schematic drawings of MB development. Side views of MB axon projections and cross-sections of the pedunculus are shown: \( \gamma \) (gray), \( \alpha'\beta' \) (magenta), and \( \alpha\beta \) (green). [B–D] Frontal projections of posterior (top) and anterior (middle) regions of the MBs. \( \alpha\beta \) neurons are marked by mCD8-GFP expression (c739-Gal4, green), and neurites of all MB are visualized by Dlg (magenta). Bottom panels show medial (side) views of 3D-surface rendered \( \alpha\beta \) neurons. (B) In nrg14; P[nrg_wt] control animals, axons of all three MB neuron subtypes project through the pedunculus (arrows) into vertical and medial MB lobes (arrowheads). (C and D) In nrg mutant animals carrying either a mutation in the extracellular domain (B; nrg849) or lacking the Nrg180-specific C terminus (C; nrg14; P[nrg180_C]), axons of \( \alpha\beta \) (green) and \( \alpha'\beta' \) (magenta/white) neurons fail to project into the pedunculus and accumulate in ball-like structures in the posterior brain ventral to the calyx (asterisks). \( \alpha\beta \) and \( \alpha'\beta' \) axons remain segregated (top, arrowheads). Axons of \( \gamma \) neurons (middle, arrowheads) still form a medial lobe that is often thinner compared with controls. (E) Frontal projections of entire MBs of an nrg849 mutant in which individual \( \alpha\beta \) neurons are labeled by mCD8-GFP (201Y-Gal4) using a flip-out approach. \( \alpha\beta \) axons are marked with FasII (red), neuropil with Dlg (blue). Bars, 20 \( \mu \text{m}. (F) Schematic model of axonal projections in nrg14; P[nrg180_C] mutant animals.
by the functional disparity and unique requirements of \( \gamma, \alpha'\beta' \)
and \( \alpha\beta \) neurons for olfactory memory acquisition, storage, and retrieval (Krashes et al., 2007; Blum et al., 2009; Trannoy et al., 2011; Qin et al., 2012). Similarly, during establishment of complex neuronal circuitry in vertebrates, axons of different identity and function are known to interact (Gallarda et al., 2008; Chen et al., 2012; Nishikimi et al., 2013; Schmidt et al., 2014), but the underlying molecular mechanisms remain largely unknown. Cell adhesion molecules (CAMs) represent likely candidates to establish and regulate the cell–cell contacts necessary to control axonal intercalation.

To address these questions, we focused on the Ig-family CAM Neuroglian (Nrg), which has been shown to be essential for MB development (Strauss and Heisenberg, 1993; Carhan et al., 2012; Nishikimi et al., 2013; Schmidt et al., 2014), but the general importance of L1CAM Neuroglian (Nrg), which has been shown to be essential for MB development (Goossens et al., 2011), the precise cellular and molecular requirements have not been addressed. To address these questions and to identify potential regulatory mechanisms, we first compared mutations affecting extracellular adhesion, \( nrg^{849} \) (Goossens et al., 2011), with mutations disrupting intracellular protein–protein interactions. To generate specific intracellular mutations, we used a genomic rescue approach (Venken et al., 2009) that allows expression of modified versions of Nrg at endogenous levels in the background of the embryonic-lethal \( nrg^{14} \) null mutation (Enneking et al., 2013). Importantly, in \( nrg^{14} \)-null mutant flies rescued by a wild-type Pacman construct (\( nrg^{14}; P[nrg\_wt] \)), all MB axons project through the pedunculus (Fig. 1 B, arrow) to the anterior part of the brain, where they branch and project into vertical and medial lobes indistinguishable from controls (Fig. 1, B, G, and H). In contrast, in \( nrg^{849} \) mutants and \( nrg \) mutants lacking the Nrg\(^{180}\)-specific intracellular tail (\( nrg^{180}; P[nrg^{180}\_AC] \); see Fig. 4 H), both \( \alpha'\beta' \) and \( \alpha\beta \) axons fail to project into the pedunculus despite the presence of \( \gamma \) lobes (Fig. 1, C–H). Strikingly, \( \alpha'\beta' \) and \( \alpha\beta \) axons remained segregated and formed two separate ball-like structures ventral to the MB calyx (Fig. 1, C and D). These data demonstrate that protein–protein interactions mediated by the cytoplasmic tail domain of Nrg\(^{180}\) are essential for MB development, indicating that intracellular interactions directly contribute to functional properties of Nrg in vivo despite being dispensable for homophilic adhesion in cellular assays (Hortsch et al., 1995). The \( nrg^{849} \) mutant phenotype has previously been described as axon stalling (Goossens et al., 2011). However, based on the close phenotypic resemblance with mutations in the \( rac \) family of actin regulators that are due to defects in MB axon guidance (Ng et al., 2002), we next analyzed the projection patterns of individual axons. We labeled individual \( \alpha\beta \) axons in \( nrg^{849} \) mutants using a flip-out approach (Gordon and Scott, 2009). GFP-labeled mutant \( \alpha\beta \) axons projected through the area of aberrant \( \alpha'\beta' \) axons (Discs Large \( \Delta Lg \) positive, Fasciclin II \( \Delta FasII \) negative) into the ball-like \( \alpha\beta \) structure (\( \Delta FasII \) positive) but failed to grow anteriorly (Fig. 1 E). Instead, the axons grew in circles without leaving the aberrant FasII-positive area (Fig. 1 E). Thus, the observed phenotypes likely represent a failure of \( nrg \) mutant axons to enter the pedunculus and are consistent with an axon guidance but not with an axon stalling phenotype (Fig. 1 F).

We next addressed whether the observed \( \alpha\beta \) and \( \alpha'\beta' \) projection phenotypes were potentially due to failed lobular innervation of \( \gamma \) neurons during larval development, which precedes \( \alpha\beta \) and \( \alpha'\beta' \) development (Fig. 1 A and Fig. S1, A–C). In contrast to a prior report (Goossens et al., 2011), we observed severe axon projection defects of \( \alpha'\beta' \) neurons resulting in posterior ball-like structures in both the extra- and intracellular \( nrg \) mutations at the third instar larval stage (Fig. S1, D–F and G).

\((G \text{ and } H)\) Quantification of aberrant ball-like projections of \( \alpha\beta \) \((G)\) or \( \alpha'\beta' \) \((H)\) neurons. Phenotypes were assayed using FasII \( \Delta FasII \) or Trio \( \Delta FasII \). \( n = 60, 46, 44, \text{ and } 54 \text{ \((G)\) and } n = 26, 28, 24, \text{ and } 34 \text{ \((H)\), in the respective order of the genotypes indicated.} \)
Importantly, analysis of \(\gamma\) axon projections using a specific GAL4-driver line (NP21) demonstrated only minor alterations in lobe morphology in \(nrg^{80}\) mutants and no defects in \(nrg^{14}\;\text{P}[nrg^{180};\Delta C]\) mutants (Fig. S1, D–F, H, and I).

**Nrg enforces axon guidance into the MB pedunculus and lobes**

To address potential functions of Nrg that may be masked by the hypomorphic nature of our extra- and intracellular mutations, we next analyzed MB neurons lacking all Nrg using the mosaic analysis with a repressible cell marker (MARCM) technique (Lee and Luo, 1999). Axons of \(nrg^{14}\) single cell mutant clones never failed to grow out or to enter the pedunculus, and we observed only minor branching defects in agreement with prior observations (Fig. 2, A–C, J; Goossens et al., 2011). To address whether Nrg is potentially required for the coordination of larger population of axons or for the interaction between axons of different identity, we next generated NB clones that...

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Figure 2. **Nrg controls MB axon tract choice.**  
(A–I) MARCM analysis of \(nrg^{14}\) mutants. Bars, 10 \(\mu\)m. (A–C) Frontal projections of control and \(nrg^{14}\) mutant single-cell clones. Absence of \(nrg^{14}\) in individual MB neurons does not cause obvious alteration of axonal projections. (D–F) Frontal projections of control and \(nrg^{14}\) \(\alpha\beta\) NB clones (NBc). The majority of \(nrg^{14}\) \(\alpha\beta\) NBc do not show an axonal phenotype (E). (F) Example of an \(nrg^{14}\) \(\alpha\beta\) NBc in which axons fail to project into the pedunculus and form circular projections in the posterior brain. (G–I) Large control and \(nrg^{14}\) NBc that include either \(\alpha\beta\) and \(\alpha\beta\) (H) or all three MB subtypes (G and I). Top panels show frontal projections of the entire MB. Bottom panels show medial (side) views of the NBc marked by GFP (green) and Dlg (blue; G and H) or FasII (red; I). In contrast to controls, \(nrg^{14}\) mutant \(\alpha\beta\) and \(\alpha\beta\) axons but not \(\gamma\) axons (I) project aberrantly straight to the \(\alpha\) lobe tip, bypassing the MB pedunculus and lobes (asterisks). (J) Quantification of MARCM phenotypes (\(n = 31, 11, 5, 131, 9,\) and 14, in the respective order of the genotypes indicated). (K) Schematic drawing of wild-type and \(nrg^{14}\) mutant axon trajectories in G–I.
either included only αβ neurons, both α′β′ and αβ neurons, or all three subtypes of MB neurons (Fig. 1 A). Interestingly, the majority of αβ NB clones did not show any alteration of axonal projections (Fig. 2, D, E, and J). However, in ~20% of these clones, we observed defects including the formation of ball-like structures below the calyx resembling the phenotype of hypomorphic nrg mutations (Fig. 2, F and J). In contrast, 78% of NB clones that included α′β′ neurons in addition to αβ neurons showed striking defects in MB development, with mutant α′β′ and αβ axons projecting straight to the tip of the α-lobes. In these cases, axons take a shortcut and circumvent their normal path through the pedunculus and the lobes (Fig. 2, G–K). Interestingly, mutant γ axons projected appropriately through the pedunculus to the lobes (Fig. 2 I). These data indicate that Nrg is not required in single axons navigating into the MB structure but is required within populations of α′β′ and/or pioneering αβ axons that likely mediate an interaction between these two distinct axonal populations.

### Dynamic expression of Nrg during MB development

To gain insights into how Nrg coordinates the guidance of α′β′ and αβ axons into the pedunculus, we next examined the expression pattern of Nrg during MB development. At late larval stages when the first α′β′ axons intercalate in between γ neurons at the center of the pedunculus (Fig. 1 A), we observed low levels of Nrg in γ axons but high levels within the axons of α′β′ neurons especially at the interface with the γ axons (Fig. 3 A). During early pupal development (4 h after puparium formation [APF]), Nrg is down-regulated in α′β′ axons and now highly expressed in c305a-Gal4-negative αβ axons. In late pupal and adult stages, Nrg is down-regulated in α′β′ axons but remains expressed at high levels in αβ and at lower levels in γ axons.
assess the requirements of these domains, we analyzed genomic rescue constructs carrying domain-specific deletions (see Materials and methods; Enneking et al., 2013). Importantly, all constructs rescued the embryonic lethality associated with the nrg14-null mutation, enabling analysis of adult MBs. It has been previously demonstrated that the C-terminal PDZ-interacting domain can bind to the MAGUK protein Polychaetoid (Pyd) in vitro, and genetic interaction studies indicated relevance of this interaction for MB development (Goossens et al., 2011). However, rescue constructs lacking the core residues of the C-terminal PDZ-interacting domain (TYV) that mediate binding to Pyd completely restored MB development, thus excluding an essential requirement of this domain (Fig. 4, A, B, and G). In contrast, absence of the Ankyrin interaction domain (FIGQY) in the neuronal isoform Nrg180 but not in Nrg167 isoform led to the formation of aberrant ball-like axon projections in the posterior brain (Fig. 4, C, D, and G). Strikingly, we observed an identical phenotype when the FERM–protein interaction domain was deleted (FERM; deletion of amino acids 1156–1166; Fig. 4, F). Together these data demonstrate that Nrg is required to guide MB axons into the pedunculus and lobes. The dynamic expression pattern suggests that Nrg potentially controls selective adhesion between populations of axons of different identity.

The intracellular FIGQY and FERM domains are required for MB development

Based on the striking phenotype caused by the partial deletion of the cytoplasmic tail of Nrg (Fig. 1, D, G, and H), we next aimed to identify the essential intracellular protein domains and their potential interaction partners. The C terminus of Nrg contains three major intracellular motifs: a FERM-interacting domain providing a potential link to the actin cytoskeleton shared by the neuronal (Nrg180) and nonneuronal isoform of Nrg (Nrg167), isoform-specific Ankyrin-interacting domains (FIGQY), and an Nrg180-specific PDZ-interacting domain (Fig. 4 H). To directly assess the requirements of these domains, we analyzed genomic rescue constructs carrying domain-specific deletions (see Materials and methods; Enneking et al., 2013). Importantly, all constructs rescued the embryonic lethality associated with the nrg14-null mutation, enabling analysis of adult MBs. It has been previously demonstrated that the C-terminal PDZ-interacting domain can bind to the MAGUK protein Polychaetoid (Pyd) in vitro, and genetic interaction studies indicated relevance of this interaction for MB development (Goossens et al., 2011). However, rescue constructs lacking the core residues of the C-terminal PDZ-interacting domain (TYV) that mediate binding to Pyd completely restored MB development, thus excluding an essential requirement of this domain (Fig. 4, A, B, and G). In contrast, absence of the Ankyrin interaction domain (FIGQY) in the neuronal isoform Nrg180 but not in Nrg167 isoform led to the formation of aberrant ball-like axon projections in the posterior brain (Fig. 4, C, D, and G). Strikingly, we observed an identical phenotype when the FERM–protein interaction domain was deleted (FERM; deletion of amino acids 1156–1166; Fig. 4,
E and G). Thus, two distinct and potentially independent intracellular Nrg protein–protein interaction motifs are essential for MB development in vivo.

Nrg-Ankyrin2 (Ank2) and Nrg-Moesin associations control MB axon guidance

Phosphorylation of the tyrosine residue within the FIGQY motif negatively regulates the binding of L1 protein family members to Ankyrins (Garver et al., 1997; Tuvia et al., 1997). This effect can be mimicked by specific amino acid substitutions, which alter binding to Ank2 and change the mobility of L1CAM in vitro (Gil et al., 2003) or of Nrg in axons in vivo (Enneking et al., 2013). Similar to nrg<sup>14</sup>; P[nrg<sup>180_ΔFIGQY</sup>], mutant animals, a point mutation abolishing the Nrg–Ank2 interaction (YA) failed to rescue MB development (nrg<sup>14</sup>; P[nrg<sup>180_YA</sup>]; Fig. 4 G). In contrast, a point mutation inducing a constitutive Nrg–Ank2 interaction by rendering the tyrosine nonphosphorylatable (YF) efficiently restored MB axon projections into the pedunculus and the anterior lobes (nrg<sup>14</sup>; P[nrg<sup>180_YF</sup>]; Fig. 4, F and G). Interestingly, in YF mutants we observed minor defects in α lobe tip innervation and a partial fusion of the β lobes from the two brain hemispheres, which indicates that a dynamic regulation of the Nrg–Ank2 interaction is essential for normal lobe morphogenesis (Fig. 4 F; and Fig. S2, A–C). To independently test the requirement of a cytoplasmic Nrg–Ank2 association, we performed genetic interactions assays. The ank2 allele nrg<sup>305</sup> significantly reduced expression of both Nrg isoforms (Fig. 5 E) and caused MB lobe formation defects in 65% of brain hemispheres but only mildly affected axonal projection into the pedunculus (Fig. 5, A and D). Strikingly, removal of one copy of ank2 (using the ank2-null allele ank2<sup>518</sup>) in hemizygous nrg<sup>305</sup> mutant animals resulted in a dramatic enhancement of the phenotype, with 80% of αβ axons now failing to enter the pedunculus (Fig. 5, C and D). Because the ank2<sup>518</sup> allele did not impair MB development in heterozygosity (Fig. 5, B and D), these data are consistent with the Nrg–Ank2 interaction contributing to MB axon guidance (Fig. 5 J).

We used a similar approach to identify potential binding partners of the Nrg FERM–protein interaction domain. Prime candidates are proteins of the ERM protein family that are represented by a single member in the Drosophila genome, Moesin (McCartney and Fehon, 1996; Adams et al., 2000). However, it has been demonstrated that Nrg can also be present in a complex with the related 4.1 protein family member Coracle (Genova and Fehon, 2003). Therefore, we tested the requirements of both Moesin and Coracle for Nrg-dependent MB development using previously characterized RNAi constructs (Ramel et al., 2013; Gardiol and St Johnston, 2014). While MB-specific RNAi-mediated knockdown of Coracle did not result in any MB defects, knockdown of Moesin resulted in lobe projection defects (Fig. 5, G and I), which indicates that Moesin is required for MB development. Indeed, knockdown in MB neurons of Moesin but not of Coracle in hemizygous nrg<sup>305</sup> mutant animals resulted in a striking enhancement of the nrg<sup>305</sup> mutant phenotype, with almost 100% of αβ axons failing to enter the pedunculus (Fig. 5, H and I). Together, these data identify Moesin and Ank2 as likely intracellular interaction partners of Nrg during MB axon guidance (Fig. 5 J).

Trans-axonal control of pedunculus and lobe formation

Based on the dynamic expression of Nrg at the border between ingrowing and substrate axons, we hypothesized that Nrg acts as a homophilic CAM to mediate axon–axon interactions during pedunculus entry. To test this hypothesis and to investigate potential cell type–specific requirements of the different Nrg domains, we used the UAS-Gal4 system to express wild-type Nrg<sup>180</sup> selectively in either αβ’ or γ neurons in the background of the domain-specific nrg mutants. In these animals, wild-type Nrg<sup>180</sup> will be present only in substrate (γ) or ingrowing axons (αβ’) while all other MB neurons express mutant Nrg. This enables a direct analysis of cell type–specific axo–axonal interactions mediated between wild-type and mutant Nrg proteins. In nrg<sup>14</sup>; P[nrg<sup>180_ΔFIGQY</sup>], expression of wild-type Nrg<sup>180</sup> in ingrowing αβ’ neurons was sufficient to rescue pedunculus entry and lobe formation of αβ’ axons (Fig. 6, A–C and E). Strikingly, in these animals we also observed an almost complete rescue of αβ axons that only express mutant Nrg lacking the FIGQY domain (Fig. 6, A’–C’ and F). Similarly, expression of wild-type Nrg<sup>180</sup> in γ neurons was sufficient to rescue projections of mutant αβ’ neurons (Fig. 6, D and E). These data demonstrate that the presence of wild-type Nrg<sup>180</sup> in either substrate or ingrowing axons is sufficient to compensate for the absence of the Nrg FIGQY protein interaction motif within the interacting axonal population and indicates that Nrg acts as a homophilic CAM during these axo–axonal interactions. Interestingly, in animals expressing wild-type Nrg<sup>180</sup> only in γ neurons, we also observed a partial rescue of αβ projections into the pedunculus, but the axons failed to innervate αβ lobes (Fig. 6, D’ and F). Analysis of the axonal projections within the pedunculus revealed a severe perturbation of axonal layer organization, with mutant αβ neurons now directly contacting wild-type Nrg<sup>180</sup>–expressing γ neurons (Fig. 6, D, arrow), a phenotype never observed in control animals (Fig. 6 A). Thus, mutant αβ axons likely used wild-type Nrg-expressing γ axons as a substrate to enter the pedunculus. However, at the end of the pedunculus these mutant αβ axons failed to use the nrg<sup>14</sup>; P[nrg<sup>180_ΔFIGQY</sup>] mutant αβ’ axons (Fig. 6, D and D’), as a template and therefore could not form αβ lobes. These data are consistent with the two axonal populations also interacting in an Nrg-dependent manner at this choice point during lobe development.

We next addressed whether a similar trans-axonal rescue is possible in animals lacking the intracellular FERM protein interaction domain of Nrg. Surprisingly, and in contrast to nrg<sup>14</sup>; P[nrg<sup>180_ΔFIGQY</sup>] mutant animals, expression of Nrg<sup>180</sup> in either ingrowing (αβ’) or substrate (γ) neurons in nrg<sup>14</sup>; P[nrg<sup>180_ΔFERM</sup>] mutant animals failed to restore the αβ’ or αβ axonal projection patterns (Fig. 6, G–I, G’–I’, K, and L). However, expression of Nrg<sup>180</sup> in all MB neurons almost completely restored MB development with only minor αβ lobe defects (Fig. 6 J, J’, K, and L). Thus, in contrast to the FIGQY domain, the FERM protein interaction domain is required simultaneously in both ingrowing and substrate neurons to allow pedunculus entry of αβ’ and αβ axons.
We then analyzed the cell type–specific requirements of extracellular adhesion using the nrg\textsuperscript{S213L} mutation, which causes an S213L exchange within the second Ig domain. It was previously reported that this mutation completely abolished Nrg-dependent homophilic cell–cell interactions in a Drosophila S2 cell aggregation assay (Goossens et al., 2011). However, a complete loss of adhesive properties of the Nrg\textsubscript{S213L} protein in vivo is not consistent with the observation that nrg\textsuperscript{S213L} mutant animals survived to adulthood while nrg\textsuperscript{S213L}–null and nrg\textsuperscript{S213L}\textsuperscript{–splice mutants} that completely lack the extracellular Ig domains 3 and 4 die as late embryos (Bieber et al., 1989; Enneking et al., 2013). Interestingly, the potential analogous human L1CAM disease mutation H210Q (Jouet et al., 1994; Vits et al., 1994) reduces homophilic L1-L1 adhesion but efficiently binds to wild-type L1 protein (Castellani et al., 2002). To address potential association between Nrg\textsubscript{S213L} and wild-type Nrg, we performed S2 cell aggregation assays using transient transfection of fluorescently tagged proteins. While expression of Nrg lacking Ig domains 3/4 did not induce cell cluster formation, we observed efficient clustering of cells expressing GFP-tagged Nrg\textsubscript{S213L} (Fig. S3). This indicates at least partial homophilic binding activity consistent with the hypomorphic nature of the nrg\textsuperscript{S213L} mutation in vivo. In addition, we observed efficient association between Nrg\textsubscript{S213L} and wild-type Nrg\textsuperscript{180}-expressing cells, demonstrating that mutant and wild-type proteins can form functional homophilic interactions (Fig. S3). Based on these results, we next tested in vivo whether formation of a trans-axonal...
Figure 6. Trans-axonal control of pedunculus and lobe formation. (A–D) Frontal projections of posterior (top) and anterior regions (middle) of MBs marked by Trio (magenta; αβ', high; and γ, low). Bottom panels show cross-sections of the pedunculus stained for Trio (magenta) and Dlg (green). Bars: (top) 20 µm; (bottom) 2.5 µm. (A–D) Top panels show frontal projections of entire MBs marked by FasII (green; αβ, high; and γ, low). Schematics summarize axonal projection phenotypes. Bars, 20 µm. (A) In control nrg14; P[nrg_wt] animals, Trio-positive axons of αβ' and γ neurons project into anterior lobes. Within the pedunculus, γ, αβ', and αβ axons are clearly segregated into distinct concentric layers. (A') αβ axons form medial and vertical lobes. (B) In nrg14; P[nrg180_\text{FIGQY}] mutant animals, αβ' axons fail to project into the pedunculus and form aberrant ball-like projections in the posterior brain. Only γ neurons (also Trio positive; imaged at higher gain settings compared with controls) form anterior lobes. (B') αβ axons fail to form anterior lobes and form aberrant projections in...
Cooperative control of Nrg-mediated axo-axonal interactions

Based on the trans-axonal rescues of nrg14; P[nrg180\_FIGQY] mutants, we hypothesized that a major function of the Ank2 interaction may be clustering of Nrg, a feature that in principle can be accomplished with equal efficacy from either side of a trans-axonal interaction. If Nrg mediated axon–axon interactions depend on the formation of Nrg clusters, we would predict intragenic complementation between the three nrg mutations despite their unique cell type-specific requirements. Strikingly, while we observed identical phenotypes for all three mutations when homo/hemizygous (Fig. 8, A–C, and G), all trans-heterozygous combinations restored pedunculus entry and at least partially rescued lobe formation (Fig. 8, D–G). These results provide strong evidence that multimeric clusters mediate Nrg function in vivo. These data further demonstrate that the Nrg\_FERM protein is functional and that the intracellular FIGQY and FERM domains act independently of each other. The observed lobe formation defects in transheterozygous nrg14 and nrg14; P[nrg\_FERM] mutants were consistent with the more essential requirements of these two protein domains (Fig. 8, F and G). Together, these data demonstrate that the extra- and intracellular Nrg protein–protein interaction domains act in a cooperative manner during the cell type-specific axon–axon interactions necessary for the establishment of MB architecture (Fig. 8 H).

Discussion

Our combined analysis using targeted, domain-specific mutations of the Drosophila L1CAM homologue Nrg with cell type–specific rescues enabled us to unravel the cellular mechanisms controlling MB development and to gain insights into the general molecular mechanisms underlying CAM-mediated cell adhesion and axon guidance in vivo. At the cellular level, we provide evidence for the potential presence of an attractive signal at the tip region of MB lobes that guides MB axons to the anterior brain. Axon–axon interactions mediated by Nrg are necessary to enforce guidance of α’β’ and then αβ neurons through the pedunculus and along the lobes to their target to establish the characteristic layered and lobular organization of the MBs essential for learning and memory. At the molecular level, our data suggest that intracellular association with Ank2 and Moesin is independently required for the establishment of functional trans-axonal Nrg complexes and MB axon guidance in vivo. Our results demonstrate that CAM-mediated axon–axon interactions are tightly controlled by intracellular protein–protein interactions and enable the establishment of complex layered and lobular neuronal circuit architecture.

Nrg controls MB axon guidance

The analysis of MB axons lacking all Nrg revealed striking insights into the mechanisms controlling MB assembly. Our data show that Nrg is not essential for neurite extension or axon pathfinding of individual neurons or of small populations of neurons of equal identity. However, as soon as nrg mutant NB clones included neurons of two identities, we observed a dramatic alteration of axon trajectories. Instead of entering the pedunculus and following the lobe pathways, mutant axons projected directly to the final target, the tip of the αα’ lobes. Together with our cell type–specific rescue data, these results indicate that Nrg is essential to mediate axon–axon interactions between axon populations of different identities to enforce and enable guidance through the MB structure. Furthermore, these data indicate that αα’ lobes are the likely source of a long-range chemotactically axon guidance signal. However, an alternative explanation may be that the shortcut pathway simply represents a permissive default trajectory. Wnt signaling represents a prime candidate to mediate MB axon guidance because it has been implicated in anterior–posterior guidance in both invertebrates and vertebrates (Lyuksyutova et al., 2003; Yoshikawa et al., 2003) and because the wnt5 mutant phenotype shares similarities with the posterior brain. (C) Expression of wild-type Nrg180 in α’β’ neurons of nrg14; P[nrg180\_FIGQY] mutants restores anterior projections of α’β’ neurons. Minor perturbations of axonal layer organization are evident in the pedunculus. (C’) In these animals, projections of αβ mutant axons are also efficiently rescued and αβ lobes form next to the wild-type Nrg180-expressing α’β’ lobes (asterisk in C). (D) Expression of wild-type Nrg180 in γ neurons of nrg14; P[nrg180\_FIGQY] mutants also rescues αβ’ projections. Pedunculus cross-sections reveal aberrant organization of axonal layers, with mutant αβ’ axons inappropriately in contact with γ axons (arrow). (D’) In these animals, αβ’ axons grow into the pedunculus to the pedunculus divide (heel, arrow) but fail to form medial or vertical lobes (note the altered appearance of α’β’ lobes in D due to the absence of αβ’ lobes, indicated by the asterisk). (E) Quantification of α’β’ phenotypes (n = 24, 55, 18, and 30, respectively, in the order of the genotypes indicated). (F) Quantification of αβ’ phenotypes (n = 44, 61, 69, and 36, respectively, in the order of the genotypes indicated). (G-J) Frontal projections of entire MBs. (G and G’) In nrg14; P[nrg\_FIGQY] mutant animals, axons of α’β’ and αβ’ neurons form aberrant ball-like projections in the posterior brain and fail to form anterior lobes. (H and H’) Expression of wild-type Nrg180 in α’β’ neurons of nrg14; P[nrg\_FIGQY] mutants does not rescue the MB phenotype. (I and I’) Expression of wild-type Nrg180 in γ neurons of nrg14; P[nrg\_FIGQY] mutants does not rescue the MB phenotype. (J and J’) Expression of wild-type Nrg180 in all MB axonal projections rescues axonal projections. Bars, 20 μm. (K) Quantification of the α’β’ phenotypes (n = 44, 61, 28, 31, and 24, respectively, in the order of the genotypes indicated). (L) Quantification of the αβ’ phenotypes (n = 102, 82, 65, 48, and 30, respectively, in the order of the genotypes indicated).
nrg MB phenotype (Grillenzoni et al., 2007). Uncoupling extracellular guidance signaling from the force-generating cytoskeletal machinery by mutating rac genes also resulted in a failure of axons to enter the pedunculus (Ng et al., 2002), which indicates that pedunculus entry represents a key choice point for αβ and αγ MB axons.

Homophilic Nrg-Nrg complexes control axo-axonal interactions

At the molecular level, our study provides mechanistic insights into the in vivo requirements of protein–protein interaction domains of CAMs during contact-dependent axon guidance. We propose a three-step process necessary for the formation of functional Nrg complexes during axo-axonal interactions.

First, adhesive contact is established by a homophilic Nrg interaction between ingrowing (αβ) and substrate (γ) axons. We provide evidence that establishment of this inter-subtype axonal interaction requires active competition for binding partners and directly correlates with the adhesive properties of Nrg. Similar to the potential analogous human L1CAM disease mutation H210Q (Jouet et al., 1994; Vits et al., 1994; Castellani et al., 2002), and in contrast to a prior report (Goossens et al., 2011), we demonstrate that mutant NrgS213L (nrg849) only partially impairs extracellular adhesion and can efficiently bind to wild-type Nrg (Nrgwt). Our cell type–specific rescues demonstrated that expression of Nrg wt in ingrowing but not in substrate neurons restores pedunculus entry of αβ' axons in nrg849 mutants. Thus, Nrgwt of ingrowing axons can interact with and resolve axo-axonal adhesion mediated by NrgS213L present on substrate axons. In contrast, in the reverse case mutant NrgS213L on ingrowing axons cannot dissolve the strong adhesive connections between substrate neurons that are mediated by Nrgwt. Identical mechanisms also control the interactions between αβ' and αβ MB neurons (Fig. S4). Consistent with this hypothesis, we
inter-axonal adhesion indicates that differential adhesion may not only mediate force-generating interactions but also contribute to the segregation of subtype-specific axonal population analogous to the adhesion-dependent sorting of synaptic fascicles observed in the *Drosophila* visual system (Schwabe et al., observed a differential and dynamic expression of Nrg during MB development, with the highest levels of Nrg always present in the ingrowing axonal population (first α’β’ then αβ) especially at the boarder to the substrate axons (first γ then α’β’). The precise control of Nrg levels that likely reflect the strength of inter-axonal adhesion indicates that differential adhesion may not only mediate force-generating interactions but also contribute to the segregation of subtype-specific axonal population analogous to the adhesion-dependent sorting of synaptic fascicles observed in the *Drosophila* visual system (Schwabe et al.,
Our data are consistent with prior studies speculatting that the later born axonal populations (αβ) follow pioneer tracts of different identity (α’β’) during MB lobe formation (Wang et al., 2002; Boyle et al., 2006; Bates et al., 2010; Shin and DiAntonio, 2011). We now identify essential requirements for trans-axonal Nrg interactions between γ-α’β’ and α’β-αβ neurons, respectively, during pedunculus entry and lobe formation. This is best highlighted in the γ cell type–specific rescue experiments in nrg14::P[nrg180_ΔFIGQY] mutants (Fig. 6, D, D’, and F). In these experiments, the larval γ lobes (before pruning) that expressed wild-type Nrg served as a substrate for α’β’ axons to project into the pedunculus and likely the lobes as well. Consistent with the requirement for a functional Nrg–Ank2 association on at least one side of the axon–axon interactions, these mutant α’β’ axons could not serve as a functional substrate for mutant αβ axons. αβ axons likely used γ axons aberrantly to grow into the pedunculus but failed to grow into lobes due to the absence of a wild-type Nrg substrate. Based on these results, it is interesting to speculate that pioneering γ neurons use glia cells as a substrate for the initial projections into anterior lobes. Indeed, we observed small alterations in γ projections in nrg180 and nrg14::P[nrg180_ΔFERM] mutants that affect both the neuronal and the glial Nrg isoform (Nrg180 and Nrg167, respectively) but not in nrg14::P[nrg180_ΔFIGQY] mutants, which is consistent with the Nrg–Ank2 association being sufficient on either side of the Nrg–Nrg interface.

In addition, the disruption of pedunculus architecture in nrg14::P[nrg180_ΔFIGQY] mutant animals expressing Nrg180 only in γ neurons (Fig. 6 D) indicates that Nrg participates in the establishment and maintenance of cell type–specific axonal layer organization. However, additional CAMs must contribute to axonal subtype segregation, as we observed a clear segregation based on axonal identity in all hypomorphic nrg mutants (Fig. 1, C and D). Dscam and FasII are expressed in subsets of MB neurons and required for MB development (Kurusu et al., 2002; Wang et al., 2002; Zhan et al., 2004). An attractive model would be that these CAMs act cooperatively during the establishment and maintenance of layered and lobular MB organization. Interestingly, analysis of CAM expression patterns in the fasciculus retroflexus identified a layer-specific and differential localization of all four vertebrate homologues of Nrg (L1, CHL1, NrCAM, and Neurofascin; Schmidt et al., 2014), which indicates potential conserved functions during axon–axon interactions that establish complex neuronal circuitry.

Finally, our data provide potential mechanistic insights into the molecular basis of the neurodevelopmental defects observed in L1 syndrome patients: partial agenesis of the corpus callosum (AgCC) and spinocerebellar projection defects (Wong et al., 1995). In patients with AgCC, callosal axons fail to cross the midline and instead form aberrant ipsilateral tracts partially maintaining topographic organization (Tovar-Moll et al., 2007). During normal development, callosal projections are established in a sequential manner depending on axon–axon interactions between axonal populations of different identities (Koester and O’Leary, 1994). Based on our data, an attractive hypothesis would be that decreased extracellular interactions between axons of different identity result in a failure to efficiently intercalate and project to appropriate targets on the contralateral side of the brain.
Table 1. Primers used in this study

| Primers | Forward | Reverse |
|---------|---------|---------|
| P[acman] FERM-GalK | 5'-TCTACCTCTTCTACATCATCTGTGATATCAGGAGCATTGAGCGG | 5'-CTTCCGGAATAATCCGCGCTGGGACCTGGCGGACGGGTGATTCG |
| P[acman] ∆FERM | 5'-TGGGCTGTCCTTCCATCTTCTATCATCAGTATATACTGCTGATTCAACGCCGAGGCGGACATCTGGAGCACTGGACATCTGGACGTATTGG | 5'-TGATAGGGCCATTGCTGGTGATTCG |
| pUAST-Nrg [2513L] | 5'-CGGATTCTTCTATGCGTGTGCACCTCGTGTCCCGACAGCG-3' | 5'-CTCGGAAACACCCAGTCCAGAATGACTGACAAAGGATAGTTCG-3' |

Materials and methods

Fly stocks

All flies were maintained at 25°C on standard fly food except for RNAi experiments (29°C). The following strains were used in this study: w1118 (Control), P[w1118] (Lee and Luo, 1999), OK107-Gal4 (Lee et al., 1999), 201Y-Gal4 (αβ core and γ neurons; Gardiol and St Johnston, 2014; all from Vienna Drosophila RNAi Center (VDR)), RNAi (9788; Gardiol and St Johnston, 2014; all from Vienna Drosophila RNAi Center), P[tub-αβ,F9A] (Lee et al., 2006), Drosophila Genomics Resource Center, 1231–1302 of Nrg180 ORF), P[w1118] (null allele), tub-FRT>Gal80-FRT, hsFlp [all from the Bloomington Stock Center], N[neoFRT]19A (NP21, γ neurons; Tanaka et al., 2008; Drosophila Genomics Resource Center), 2007), UAS-Fushima and Tsujimura, 2007), and P[acman] (all from Enneking et al., 2013), P[nrg14;P[acman]-GT4], P[nrg14;P[acman]-GT4] (deletion of aa 1231–1235 in ORF of Nrg180), P[nrg14;P[acman]-GT4] (Y1235A substitution in ORF of Nrg180), P[nrg14;P[acman]-GT4] (deletion of aa 1230–1234 in ORF of Nrg180), P[nrg14;P[acman]-GT4] (Y1235A substitution in ORF of Nrg180), P[nrg14;P[acman]-GT4] (deletion of aa 1156–1166 resulting in nrg14;P[acman]-GT4>UAS-mCD8-GFP. Thinner γ lobes marked by P[nrg14;P[acman]-GT4>UAS-mCD8-GFP in the anterior brain were scored as “lobe defects.” n indicates the number of analyzed MBs. The two brain hemispheres were independently quantified. The following genotypes were used as controls: w1118 (Fig. 1 and Fig. 7, A and E), P[neoFRT]19A (Fig. 2, Fig. 7 F, and Fig. 5 D), and nrg14;P[acman] (Fig. 7 A, Fig. 5 I, and as indicated).

Generation of nrg constructs

Generation of the pUASt-nrg[2513L]-EGFP, pUASt-nrg[2513L]-mCherry, and nrg_∆FERM [pacman] constructs was performed according to Enneking et al. (2013). In brief, the full-length nrg[180] ORF was amplified and cloned into the pENTR vector via TOPO cloning (Life Technologies). Point mutations were introduced into pENTR clones using the QuikChange II site-directed mutagenesis kit (Agilent Technologies). Final constructs were shuffled into tagged pUASt vectors (Enneking et al., 2013) using Gateway technology (Life Technologies). To generate the nrg_∆FERM constructs, the wild-type nrg[pacman] construct was modified using galK-mediated recombineering as described previously (Enneking et al., 2013). aa 1156–1166 of the open reading frame of Nrg180 were first replaced with a galK encoding construct. Using negative selection, the galK cassette was exchanged using a template lacking aa 1156–1166 resulting in nrg_∆FERM. All steps were performed according to the protocol provided by the NCI Frederick National Laboratory [http://ncifrederick.cancer.gov/research/brbr/recombineeringInformation.aspx]. All cloning steps were verified by sequencing. All constructs were inserted into the attP40 landing site using site-specific integration via the phiC31 system (Bischel et al., 2007). All primers used in this study are listed in Table 1.

Cell culture, transfection, and cell aggregation assay

Schneider’s line 2 (S2) cells were maintained in complete Schneider’s medium at 25°C with air as the gas phase. Cells were transfected using X-tremeGENE HP DNA Transfection Reagent (Roche). Expression of transfected genes was allowed for 3 d. Differently transfected cell populations were mixed in 2 ml Cryo tubes (Eppendorf) and incubated on a rotator for 2 h to allow for cell aggregation. For imaging, cells were pipetted onto microscope slides and images were captured using a laser scanning confocal microscope (TSC-SP; Leica). For quantification, six
images were captured at fixed positions and quantified manually using ImageJ software.

Western blot analysis
Larval brains from wandering third instar larvae were dissected and transferred into 2x sample buffer (Life Technologies). NuPage gels (Invitrogen) were loaded with five brains per lane. Western blotting was performed according to Enneking et al. (2013). In brief, membranes were incubated overnight at 4°C with the following primary antibodies: mouse anti-Nrg3C1 (1:500), which recognizes extracellular domains of Nrg167 and Nrg14 (1:500; a gift from M. Hortsch, University of Michigan, Ann Arbor, MI; Hortsch et al., 1990); mouse anti-Nrg3 (1:500; 1:200; Hortsch et al., 1990); and mouse anti-phosphotyrosine (E7; 1:50; both from the Developmental Studies Hybridoma Bank) followed by a 2-h incubation at room temperature with secondary HRP-conjugated goat anti–mouse and goat anti–rabbit at 1:10,000 (Jackson ImmunoResearch Laboratories, Inc.).

Online supplemental material
Fig. S1 compares the wild-type MB architecture to the phenotype of nrg180; nrg167 and nrg14; [P[nrg167 △C];P[nrg180 △YF]] mutants at the end of larval development. Fig. S2 shows the axon midline crossing phenotype of ßC core neurons in nrg14; [P[nrg180 △YF]] mutant animals. Fig. S3 demonstrates the cell adhesive functions of wild-type and mutant Nrg protein using an S2 cell aggregation assay. Fig. S4 summarizes the cell type–specific requirements of the different Nrg domains. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201407131/DC1.

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Author contributions: D. Siegenthaler performed all experiments. E.M. Enneking generated constructs and fly stocks. E. Moreno generated constructs. D. Siegenthaler and J. Pielage designed experiments, analyzed data, and wrote the manuscript.

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