Introduction

Neurons extend axons and dendrites, and these neurites select specific partners for establishing interneuronal connections. In the case of interactions between hippocampal pyramidal neurons, axons attach to dendrites for their synaptogenesis, but the dendrites do not form stable contacts with each other, suggesting the presence of a mechanism to allow their selective associations. Nectin-1 (N1), an immunoglobulin domain adhesive protein, is preferentially localized in axons, and its heterophilic partner, N3, is present in both axons and dendrites; we tested their potential roles in interneurite recognition. The overexpression of N1, causing its mislocalization to dendrites, induced atypical dendrodendritic as well as excessive axodendritic associations. On the contrary, the genetic deletion of N1 loosened the contacts between axons and dendritic spines. Those actions of nectins required cadherin–catenin activities, but the overexpression of cadherin itself could not accelerate neurite attachment. These results suggest that the axon-biased localization of N1 and its transinteraction with N3 in cooperation with the cadherin machinery is critical for the ordered association of axons and dendrites.

Interneurite affinity is regulated by heterophilic nectin interactions in concert with the cadherin machinery

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Abbreviations used in this paper: CP, cytoplasmic; DIV, days in vitro; EC, extracellular.
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Their axons and dendrites were identified by immunostaining campal pyramidal neurons cultured for 4–6 d in vitro (DIV). We first examined the distribution of N1 and N3 in rat hippocampal neurites. In this study, we tested this idea and found that the trans-interaction of N1 and N3 indeed controlled the adhesion of contacts, such as dendrodendritic contacts, not stabilized.

Differential distribution of N1 and N3

Why cadherins are active only for axodendritic connections.

Some classes of molecules that have cell-binding activities are localized only in axons or dendrites, and their partners are present on the counter-neurites. For example, neurexin is expressed by dendrites, whereas its ligand, neuroligin, is localized in axons (Scheiffele, 2003). Such receptor–ligand systems should be able to facilitate the selective contacts between axon and dendrite but not those between dendrite and dendrite or axon and axon. In the case of neurexin and neuroligin, their molecular interactions have been implicated in synaptic differentiation (Scheiffele et al., 2000; Dean et al., 2003; Graf et al., 2004). Although the neurexin–neurexin interaction has been shown to promote cell adhesion (Ichtchenko et al., 1996; Nguyen and Sudhof, 1997), it remains to be defined whether these molecules are important for maintaining the physical associations between axons and dendrites.

Nectins, forming a small subfamily of Ig domain proteins, show an asymmetrical distribution in synapses (Mizoguchi et al., 2002). In the mossy fiber terminals of the hippocampus, nectin-1 (N1) is predominately localized in the presynaptic membrane, and N3 is localized in the postsynaptic membrane, whereas their CP partner l-afadin is detected in both membranes (Mizoguchi et al., 2002). Both N1 and N3 show homophilic binding abilities and can promote cell aggregation (Takahashi et al., 1999; Satoh-Horikawa et al., 2000). However, importantly, they also can bind one another heterophilically, and this heterotypic binding is much stronger than the homophilic one (Fabre et al., 2002; Yasumi et al., 2003; Martinez-Rico et al., 2005). Furthermore, nectin interactions at cell–cell boundaries promote the recruitment of cadherin molecules to these sites (Tachihana et al., 2000; Honda et al., 2003). These unique distributions and properties of nectins suggest that they may play an active role in the preferential contacts between axons and dendrites. In this study, we tested this idea and found that the trans-interaction of N1 and N3 indeed controlled the adhesion between these heterotypic neurites. Our results also explain why cadherins are active only for axodendritic connections.

Results

Differential distribution of N1 and N3 in neurites

We first examined the distribution of N1 and N3 in rat hippocampal pyramidal neurons cultured for 4–6 d in vitro (DIV). Their axons and dendrites were identified by immunostaining for dephosphorylated tau (Binder et al., 1985) and MAP2 (Caceres et al., 1984), respectively. In isolated single neurons, both N1 and N3 were detected diffusely along their neurites, but together, whether heterotopic or homotypic, if the cells express the same cadherin types (Hirano et al., 1987). In neurons, cadherins are localized in both axons and dendrites. Curiously, however, in many neurons such as hippocampal pyramidal neurons, although firm contacts between axons and dendritic spines are formed depending on cadherin activities, other types of contacts, such as dendrodendritic contacts, are not stabilized. We must ask why cadherins participate predominantly in the heterotypic (axodendritic) synaptic junctions but not in the homotypic dendrodendritic contacts even though this molecular family is in general used for linking the “like” cells. There should be some mechanisms for allowing cadherins to promote specifically axodendritic associations in these neurons.

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they displayed distinct patterns of distribution: N1 was always more abundant in axons than in dendrites, whereas N3 was equally present in axons and dendrites (Fig. 1, a, b, and i). When axodendritic contact formation began, both N1 and N3 became concentrated at early synaptic contacts formed on the tip of dendritic filopodia, overlapping with β-catenin, a representative of the cadherin–catenin complex. Concomitantly, the diffuse nectin signals disappeared from the axons (Fig. 1, c and d). Dendrites from different neurons did not form firm contacts to each other, but they occasionally happened to cross. At these dendrodendritic crossing points, N1 was not detectable (Fig. 1 e), and N3 was present around the dendrodendritic interfaces but was not particularly concentrated there (compare the faint N3 signals on these sites with those highly up-regulated at synaptic contacts on the same dendrite in Fig. 1 f). β-Catenin was detected on some of the dendrodendritic crossing points (Fig. 1, e and f) but not on all of them (<40% at 10 DIV). These dendrodendritic β-catenin accumulations became hardly detectable at later stages (e.g., at 14 DIV). In summary, N1 and N3 were preferentially concentrated at axodendritic interfaces.

Once the nectin signals had been concentrated in the synapses, it became difficult to define whether these signals were derived from axons or dendrites. To determine their localization in mature neurons accurately, we transfected neurons with N1 or N3 cDNA, cultured them for 3 wk, and observed the distributions of the exogenously introduced nectins. Because of the overexpression, excess nectin signals were not restricted to synapses but were diffusely detected along neurites, allowing us to determine which neurites expressed these nectins. In N1-transfected cultures, N1 immunofluorescence signals were detected emanating from thin neurites that migrated on the culture plate as well as from those associated with dendritic processes (Fig. S1 a, available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1). The former population of neurites was identified as axonal because of their MAP2 negativity. Closer observations of the latter revealed that the N1 signals were localized along spine-free neurites running on the dendritic shaft (Fig. 1 g), suggesting that these also were axons. The majority of the dendritic spines in these cultures were N1 negative. On the other hand, N3 immunofluorescence signals evenly delineated the entire dendritic process, showing typical arrays of spines (Fig. 1 h and Fig. S1 b), and their signals were barely detectable on MAP2-negative neurites. These findings indicate that N1 prefers to localize in axons at any developmental stage and that N3 localization becomes biased toward dendrites during development, which is consistent with the in vivo observation (Mizoguchi et al., 2002).

Effects of the overexpression of nectins on neurite patterning

To study the role of N1 and N3 showing the aforementioned differential distribution, we examined the effects of N1 or N3 overexpression in more detail by observing neurons earlier after their transfection. Neurons transfected with N1 or N3 cDNA were cultured for 5–6 d and analyzed for the expression of exogenous nectins. Western blot analysis showed that the total level of N1 or N3 in these cultures significantly increased (Fig. S1 c). Immunostaining analysis revealed that exogenous N1 (exN1) was abundant in axons, but, as a result of overexpression, its relative level in dendrites appeared to have increased (Figs. 1 i and 2 a). Intriguingly, this N1 overexpression caused abnormal neurite patterning. Nontransfected pyramidal neurons, in principle, extended axons and dendrites radially from their soma (Fig. 2 c). In N1-overexpressing neurons, however, their axons often entwined around their own dendrites (Fig. 2 a, tau). Furthermore, many of their dendrites aberrantly touched each other, giving a looplike appearance (Fig. 2 a, MAP2; see Fig. 3 f for quantification). On the other hand, axons...
of neurons with eN3, which was distributed evenly among neurites (as seen for the endogenous N3 [enN3]), did not show such abnormal migration: when their axons happened to migrate onto their own bodies, they crossed them with a simple track (Fig. 2 b, arrow). A similar crossing was observed in non-transfected neurons, suggesting that this behavior was not caused by N3 overexpression. At 14 DIV, neurons expressing eN1 again exhibited extensive intraneuronal dendritic attachments, whereas those expressing N3 did so only at a minimum level (Fig. 2 d and e). We could not accurately trace axons in these older cultures, as tau distribution lost its continuity along the axons. These observations indicate that the overexpression of N1 but not N3 induced atypical sticking between neurites.

Analysis of nectin domains responsible for abnormal neurite patterning

To determine which domain (the extracellular [EC] or CP) was critical for the aforementioned activity of nectins, we expressed the EC domain of N1 (N1-EC) or N3 (N3-EC; Fig. 3 a) in neurons and found that both constructs were not particularly effective in inducing aberrant neurite patterning (Fig. S2, a and b; available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1). Both of these molecules were detected on axons and dendrites, although N3-EC was homogenously distributed, whereas N1-EC tended to be clustered. We also expressed the CP domain of N1 (N1-CP) and N3 (N3-CP) but found no effects on neurite patterning. Both of these constructs tended to accumulate in the cell body regions, but a fraction of them also spread into neurites. Importantly, although N3-CP was widely distributed into MAP2-positive neurites, N1-CP was uniquely condensed along a single neurite extending from the soma of each neuron; these neurites had been identified as axons, as they did not react with anti-MAP2 antibodies except at the proximal region (Fig. S2, c and d). These results suggest that the CP domain of N1 was responsible for its axon-biased localization and also that this domain of N1 or its EC domain alone was not sufficient to exhibit the biological activities.

We also examined whether the aforementioned activities of nectins required the COOH-terminal afadin-binding site by using a COOH-terminus-truncated construct of N1 (N1∆PDZ) or N3 (N3∆PDZ) and found that their expression in neurons had no effect on dendrite patterning (Fig. S2, e and f). On the other hand, N1∆PDZ was still preferentially condensed in axon fibers (Fig. S2 e). These results suggest that the afadin-binding site is necessary for the activity of N1 to promote interneurite attachment, but the sorting signals for axonal localization reside elsewhere in the CP domain.

Figure 3. Effects of the expression of chimeric nectin molecules. (a) Diagram of nectin constructs used. TM, trans-membrane region. The N1-derived regions are light blue, and N3-derived regions are pink. + indicates where the two regions are fused. (b and c) Neurons transfected with N13 or N31 and triple stained for the chimeric nectin MAP2 and tau at 5 DIV. In N13 transfectants (b), N13 molecules are clustered in various regions, and dendrites and axons are strongly entangled. In N31 transfectants (c), N31 molecules are localized most abundantly in axons, and the atypical association of neurites is less extensive than in the case of N13 transfectants. (d and e) Neurons transfected with N13 or N31 and double stained for the chimeric nectin and MAP2 at 14 DIV. N13 strongly induces dendrodendritic attachments (d), and N31 only induces these weakly (e). (f) Statistical analysis of dendritic arbor pattern. Number of dendrites, dendrite length, and dendrite branch number in neurons nontransfected (Ctrl) or transfected with N1, N13, N3, or N31 were measured at 7 DIV. Histogram shows the mean plus SEM (error bars) for each sample (n = 20 for dendrite and branch number; n = 40 for dendrite length). No significant difference was found between these samples. For the right histogram, n = 20. ** P < 0.001 versus control, N3, and N31. The circle-crossing index represents the mean number of dendrites that cross the circle (40 μm in diameter) superimposed on the soma of each neuron. This index is expected to increase when dendrites turn and form loops as a result of dendrodendritic attachments. Bars, 20 μm.
To further investigate the roles of the EC or CP domains of nectins, we constructed chimeric molecules of N1 and N3, N13 and N31, by swapping their EC and CP domains (Fig. 3 a). When their transfectants were examined at 5–6 DIV, N13 (N1-EC + N3-CP) was detected on both axons and dendrites as clustered signals (Fig. 3 b), whereas the N31, having N3-EC + N1-CP, was localized more abundantly in axons (Fig. 3 c). This was reminiscent of the enN1 localization. These results support the idea that the CP domain is responsible for the axon-biased distribution of N1.

The expression of N13 caused the severe entangling of axons along their own dendrites (Fig. 3 b), as in the case of N1 overexpression. In neurons expressing N31, their axons also showed a tendency to attach to their own dendrites (Fig. 3 c), but to a lesser extent as compared with the N13 expression (i.e., those axons simply crossed dendrites in most cases in contrast with the firm tangle of axons with dendrites in N13-expressing neurons). Aberrant attachment between dendritic processes was also induced by N13 expression but not N31 (Fig. 3, d and e) and was confirmed by quantitative analysis (Fig. 3 f). These findings suggest that misexpression of the N1-EC domain, whether it is linked with its own CP domain or with the N3-CP domain, induces atypical neurite associations.

Trans-interaction between N1 and N3 in aberrant neurite association

We sought to understand how N1 misexpression induced the abnormal neurite interactions. Double immunostaining for N1 and N3 revealed that in N1-transfected neurons exhibiting atypical dendrodendritic contacts, exN1 molecules were concentrated together with enN3 at their contact sites (Fig. 2 f), suggesting that their heterophilic interactions were involved in inducing these phenotypes. In neurons transfected with N3, exN3 was unable to condense at the sites where their dendrites had happened to touch each other (Fig. 2 g). In these dendrites, exN3 was deposited along their noncontacting portions together with enN1. These observations suggest that only excess N1 molecules were able to accumulate themselves and their partner molecules into ectopic neurite contact sites and sustain their atypical associations. Whether exN1 also recruited the same nectin type remains to be determined because our antibodies to detect endogenous nectins could not distinguish between the exogenous and endogenous molecules.

To test further whether the interaction between N1 and N3 was important for the aberrant neurite sticking, we mixed N1- and N3-transfected neurons in the same cultures. When transfectants with the different nectins happened to reside next to each other, their dendritic branches became deeply intermingled (Fig. 4 a). At their contact points, the two molecules were closely colocalized. Thus, the trans-interactions between overexpressed N1 and N3 molecules induced interneuronal dendrodendritic associations, which are not generally observed in hippocampal cultures except for their simple crossing (Fig. 1, e and f). Similar cocondensation of N1 and N3 was also found at axodendritic contact sites formed between these transfectants. For example, when an axon expressing exN1 migrates on other neurons with exN3, the exN3 molecules have sharply been concentrated along the axon (Fig. 4 b). In many such cases, noncontacting portions of the recipient neuron lost the exN3 signals, suggesting that the majority of N3 molecules expressed by the cell had been accumulated at the axodendritic contact sites. All of these results support the hypothesis that the N1–N3 interaction facilitates interneurite adhesions. In addition, we examined whether the nectin overexpression also affected synapse formation by immunostaining the aforementioned mixed cultures for synaptic markers and found that at the contact sites between N1-overexpressing axons and N3-overexpressing dendrites, the distribution pattern of synaptotagmin, a presynaptic marker, was not particularly altered (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1). This result suggests that the N1–N3 interactions enhance only the affinities between neurites but not their synaptogenesis, which probably requires additional machineries such as the neuroligin–neurexin interactions.

Figure 4. Nectin interactions at neurite contact sites. (a) Dendrodendritic associations observed between different neurons and induced by nectin overexpression. Neurons were independently transfected with N1 or N3, and these cells were mixed at a 1:1 ratio, cultured for 14 d, and triple stained for exN1, exN3, and MAP2. In the pair of neurons situated next to each other [one overexpressing exN1 (single asterisk) and the other overexpressing exN3 (double asterisks)], their dendrites have almost entirely intermingled. The nontransfected neuron located at the bottom left is less extensively associated with them. Neurons with exN1 or exN3 were identified by the abundance of the respective molecules at the cell body regions. (b) Heterophilic nectin interaction at axodendritic interfaces. An axon extending from a remote neuron with exN1, located outside at the top left corner, has attached to dendrites of a neuron with exN3, which is visualized by MAP2 staining, in the culture prepared as in panel a. At their contact sites, exN3 is exclusively concentrated along the exN1-positive axon. Faint fluorescence on the neuronal body in the exN1 panel is likely a result of the nonspecific reaction of the antibodies. (c–f) Interaction of nectins at the interfaces between neurites and 293 cells. Neurons were plated onto mixed cultures of 293 cells nontransfected or transfected with N1 (c and d) or N3 (e and f), incubated for 5 d, and double stained for exN1 or exN3 and MAP2 or tau. Dendrites have strongly recruited N1 molecules derived from 293 transfectants to their contact sites (c) but have N3 ones only weakly (e). Axons recruited these nectins indiscriminately (d and f). Bars (a and b), 20 μm; (c–f) 10 μm.
Preferential trans-recruitment of N1 by dendrites

We hypothesized that the role of enN1 localized in axons might be to promote the attachment of the axons to dendrites through its trans-interactions with dendritic N3 molecules. As a step to test this idea, we asked with which of the nectin types dendrites or axons prefer to interact. As neurite–neurite interfaces do not provide sufficient resolution for this analysis, we constructed a model system: we prepared HEK293 (293) cells transfected with N1 (N1-293) or N3 (N3-293). These cells endogenously express N-cadherin, and the respective nectins were concentrated at their cell–cell boundaries (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1). We seeded neurons onto monolayers of these 293 transfectants and observed the distribution of each nectin at the interfaces between neurites and 293 cells. For accurate assessment of the specific effects of nectin expression, we used mosaic cultures of transfected and nontransfected 293 cells. Neurons extended their axons and dendrites onto the surfaces of these transfectants or nontransfected.

Immunostaining of these samples showed that when dendrites had attached onto N1-293 cells, N1 molecules derived from the N1-293 cells became intensely concentrated along the dendritic processes (Fig. 4 c). On N3-293 cells, the dendrites also recruited N3 molecules, but only faintly (Fig. 4 e; our antibodies against N1 and N3 could detect the antigens from these transfectants with similar fluorescence intensity; see Fig. S4). Thus, dendrites preferentially recruited N1 that was present on the counter–cell membranes. On the other hand, when axons had attached to N1- or N3-293 cells, these nectins were similarly concentrated along the axons, although not uniformly (Fig. 4, d and f), which is consistent with the finding that early axons expressed both N1 and N3. This result suggests that axonal N1 and N3 are ready to interact with the counter-nectins if these molecules are expressed on the surfaces of the adjacent cells. However, dendrites, which are the actual partners for axons, did not equally express these nectins and responded to them differentially, as shown above. Thus, as a result of the nonuniform distribution of N1 in neurites, a biased interacting system between dendritic N3 and axonal N1 seems to have been established (see Fig. 7).

Effects of the genetic deletion of N1 on axodendritic association

To verify the hypothesis that the N1–N3 interaction regulates axodendritic association, we examined the effects of the genetic deficiency of N1 on neurite patterning by culturing hippocampal neurons isolated from N1 knockout mice (Inagaki et al., 2005). Radial extension of axons and dendrites normally occurred in the mutant pyramidal neurons, and they did not display any aberrant patterning. However, when axodendritic contacts had begun, the mutant neurons came to exhibit atypical morphologies. Actin staining at 14–17 DIV revealed that their dendritic spines were unusually elongated or deformed, resulting in a smaller spine head (Fig. 5, a and g). In the cultures of wild-type neurons, their spine heads swelled, firmly attaching to axon...
fibers (Fig. 5 b, top). In contrast, in N1-deficient neurons, many of their dendritic spines, which exhibited filopodia-like morphology, did not associate with axons that could have been traced with their diffuse synaptotagmin signals (Fig. 5 b, bottom). All of these results suggest that the adhesive affinity between axons and dendritic spines, the major structures on pyramidal neurons to receive axonal input, was significantly reduced as a result of N1 deficiency. In more mature stages, synaptotagmin or β-catenin became concentrated onto their spine heads even in mutant neurons. Nevertheless, their signals were generally reduced, corresponding to the reduction in head size, and many of the spine or filopodial heads lost their association with synaptotagmin signals (Fig. 5, c–f). These results indicate that although N1 is dispensable for synapse formation, its absence impairs the normal process of axodendritic spine contacts and keeps them looser than usual even after their maturation.

Cooperative action of nectins and the cadherin-catenin complex
Because a role of nectins is to recruit cadherins, we tested whether the aforementioned activities of nectins involved cadherin actions. We found that whenever N1 and N3 were concentrated together at neurite contact sites, β-catenin was also recruited to these sites (see example in Fig. 6 a), and N-cadherin showed a similar response (not depicted), confirming that nectin interactions promote cadherin-mediated adhesion. To examine how much the heterophilic N1–N3 and homophilic N1–N1 or N3–N3 interactions differ in their abilities to recruit β-catenin, we prepared mixed cultures of N1- and N3-293. In the original transfectants, either N1 or N3 was concentrated at cell–cell boundaries, although β-catenin was less tightly colocalized with N3 than with N1 (Fig. S4). In the mixed cultures, three types of interfaces—N1–N1, N3–N3, and N1–N3—were formed. Triple immunostaining for N1, N3, and β-catenin in these cultures showed the clear tendency that N1 and N3 were more intensely condensed together at the heterotypic boundaries between N1- and N3-293 cells than at the homotypic boundaries, and the β-catenin level proportionally increased in those heterotypic contact sites (Fig. 6 b and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1). As a consequence, the junctional accumulation of these molecules was relatively decreased at N1–N1 or N3–N3 cell interfaces and, surprisingly, even disappeared from certain homotypic boundaries, causing the local separation of cells at these boundaries (Fig. S5). These results indicate that the homotypic and heterotypic cell boundaries compete for β-catenin recruitment and that the latter prevails.

The aforementioned observations in a model system confirmed that the trans-interaction between N1 and N3 most effectively recruited cadherin–catenin complexes to cell contact sites. We also noticed that in N1 or N3-transfected neurons, the total level of N-cadherin slightly increased approximately two times per neuron in each transfectant (Fig. S1 c), suggesting that both nectins can stabilize cadherins irrespective of their abilities to induce excessive interneurite contacts. Therefore, we tested whether an increase in cadherin-dependent adhesive-ness was sufficient to induce excessive neurite interactions by overexpressing N-cadherin in neurons. The total N-cadherin level per neuron increased two to three times in these cultures compared with untransfected cultures (Fig. 6 h). However, this N-cadherin overexpression had no effect on neurite patterning (Fig. 6, c, d, and g) even though cadherins had previously been shown to generate much stronger adhesiveness than nectins.

Figure 6. Cooperative action of nectins and N-cadherin/catenins. (a) A mixed culture of neurons transfected with N1 or N3 prepared as in Fig. 4 B and triple stained for β-catenin, exN3, and exN1 at 10 DIV. Axons expressing exN3 have migrated on a neuron with exN1, and β-catenin is concentrated together with N3 and N1 along the axons. (b) A mixed culture of 293 cells transfected with N1 or N3 triple stained for exN1, exN3, and β-catenin. Each transfectant was identified by comparing the localization of these two molecules and are marked as 1 (for N1) or 3 (for N3). A single N3 transfectant is surrounded by multiple N1 transfectants. (c and d) Neurons nontransfected (c) or transfected with Flag-tagged N-cadherin cDNA (d; Nakagawa and Takeichi, 1998) were cultured for 7 d and double stained for MAP2 and the Flag tag. (e and f) Hippocampal neurons derived from wild-type (e) or α-N-catenin knockout (KO) mice (f) were transfected with N1 and double stained for MAP2 and exN1 at 8 DIV. (g) Statistical analysis of the experiments in panels c and d. n = 20 for dendrite number and dendrite branch number; n = 40 for dendrite length. (h) Immunoblots for N-cadherin expressed in nontransfected (Ctrl) and N-cadherin–transfected (N-cad) cultures at 8 DIV. EGFP-tagged N-cadherin (asterisk; Honkawa and Takeichi, 2001) was used for transfection. The amount of the exogenous N-cadherin is about equal to or slightly lower than that of the endogenous one. As ~50% of cells were transfected, we can estimate that the level of total N-cadherin per neuron increased two to three times in the transfected neurons. (i) Statistical analysis of the experiments in panels e and f. Error bars represent SEM. **, P < 0.001 versus control. n = 20 for dendrite number and dendrite branch number; n = 40 for dendrite length. 20 neurons were used for this assay. Bars [a–d], 20 μm; [e and f] 10 μm.
(Martinez-Rico et al., 2005), indicating that a simple increase in cadherin level or surface adhesiveness was not sufficient for inducing the atypical neurite interactions.

Finally, we asked whether the cadherin–catenin adhesion system was required for the aforementioned actions of nectins. To test this possibility, we isolated hippocampal neurons from αN-catenin–deleted mutant mice in which cadherin activities were impaired (Abe et al., 2004) and transfected them with N1 cDNA. We first confirmed that mouse pyramidal neurons responded to N1 overexpression in a way similar to the rat ones (Fig. 6, e and i). Notably, when αN-catenin–deleted neurons had been used for transfection, their dendritic morphology was little affected by N1 overexpression (Fig. 6, f and i). We also found that the N-cadherin level was kept lower in αN-catenin–deficient neurons than in wild-type ones after the N1 transfection (Fig. S1 d). Thus, these results demonstrate that the cadherin–αN-catenin system was required for the actions of the nectins.

**Discussion**

We showed that N1 was preferentially localized in axons and that perturbation of its distribution by overexpression induced atypical associations between neurites. On the other hand, N3 was equally detected on both axons and dendrites, although this molecule appeared to prefer localizing to dendrites in mature neurons. Upon synaptogenesis, both N1 and N3 became concentrated together at axodendritic contact sites, whereas such condensation did not occur at the sites where dendrites crossed each other. In the 293 cell model system, the N1–N3 heterophilic interaction prevailed over the homophilic one, with more recruitment of β-catenin by the former. We also showed that dendrites more efficiently recruited N1 than N3 onto the counter–cell membranes, implying that the dendrites dominantly use their N3 molecules to interact with axons. Furthermore, N1-deleted neurons exhibited loosened associations between axons and dendritic spines. All of these results support the idea that the axon-biased localization of N1 and its trans-interaction with dendritic N3 plays a critical role in sustaining the normal association between axons and dendrites. These actions of nectins required cadherin–catenin activities. Intriguingly, however, the overexpression of N-cadherin by itself had no effect on neurite patterning. Thus, a cooperation of these heterophilic and homophilic adhesion systems is required for exerting their full activities, possibly generating unique mechanisms for linking the heterotypic pair of axonal and dendritic plasma membranes.

The overexpression of N1 resulted in the excessive association of axons and dendrites derived from the same neuron. The formation of synapses by neurons onto themselves occurs normally, whose structures are known as autapses (Bekkers and Stevens, 1991; Lubke et al., 1996). However, the overexpressed N1 appeared to have overly attracted axons and dendrites and, furthermore, induced atypical dendrodendritic contacts. Under these conditions, N1 molecules were leaked out to dendrites and ectopically condensed at dendrodendritic interfaces, recruiting enN3 to these sites. This suggests that the mislocalization of excess N1 and its interaction with N3 was a primary cause for the induction of the dendrodendritic adhesions (Fig. 7 b).

Once the level of N1 has increased in dendrites, this molecule should also be able to undergo substantial interactions with axonal N3, accounting for the excessive axodendritic associations (Fig. 7 b). This idea is supported by the observation that N13 exhibited similar effects. As N13 has the N3-CP domain, this chimera molecule should have followed the N3 distribution, ensuring ectopic localization of the N1-EC domain to dendrites. Together, our results indicate that the proper localization of N1 is important for the correct neurite interactions.

On the other hand, the overexpression of N3 had little effect. exN3 molecules did not accumulate at dendrodendritic interfaces, suggesting that these molecules cannot actively hold their attachments. It should be noted that the N3–N3 homophilic interactions are less effective in inducing cell aggregation than those of N1 (Martinez-Rico et al., 2005). We also noticed that N3 less efficiently recruited β-catenin to cell–cell contact sites compared with N1 in 293 cells; nevertheless, the total level of N-cadherin increased not only in N1- but also in N3-transfected neurons. Thus, we can speculate that this nectin can interact with the cadherin–catenin complex by itself but is unable to efficiently bring the complex into cell contact sites for some reason. Based on these observations, we suspect that N3 itself may not be a strong adhesion molecule and that it functions only significantly as a heterophilic partner for N1. Once the level of N3 has reached saturation with respect to N1, excess N3 molecules may not be able to exert additional biological effects.

Although these two nectins were differentially distributed in axons and dendrites, they were not strictly confined to either of these neurites, particularly in early neurons. Thus, we can suppose that nectins or cadherins can also be used to promote dendrodendritic adhesion (Fig. 7). However, this form of adhesion
where nectins were not concentrated, accumulation of cadherins at cell–cell contact sites (Tachibana dendritic interfaces, prohibiting their associations. at axodendritic interfaces could sweep away N1 or N3 from den- nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den- icate the heterophilic and homophilic interactions of nectins also likely occurs in neurons, and the N1–N3 interactions at axodendritic interfaces could sweep away N1 or N3 from den-
were ligated through the underlined Sall sites. To construct the expression vector for N13 and N31, we subcloned each cDNA fragment into pCAGpa using a HindIII and NheI linker. The Flag-tagged nectin CP region of N13 (pCAG-N1CP; 356–512 residues) and N33 (pCAG-N3CP; 404–545 residues) were constructed by using pCAG-Sig-pA. For the construction of pCAG-N1EC-GFP and pCAG-N3EC-GFP, cDNA fragments encoding amino acids 1–297 of mouse N11 and 1–425 of N33 were ligated into pCAG-GFP-pA, respectively. The generation of other constructs was described previously (Tanoue and Takeichi, 2004).

**Immunocytochemistry**

Cells on coverslips were fixed in 2–4% PFA in HBSS with 4% sucrose for 10–15 min at room temperature or 37°C. After treatment with 0.25% Triton X-100 in TBST (1× TBST with 0.005% Tween-20) for 5 min at room temperature, the cells were blocked with 5% skim milk in TBST at room temperature or 37°C. Primary antibodies were visualized with goat fluorochrome-conjugated secondary antibodies. The fluorochromes used were AlexaFluor350, -488, -555, -647 (Invitrogen), and Cy3 (Chemicon). Factin was visualized by use of AlexaFluor488-conjugated phallodin (Invitrogen).

**Antibodies**

Rabbit anti-N1 and anti-N3 antibodies were raised against the CP portion of mouse N11 and N33 proteins, respectively, and were affinity purified by using standard protocols. These antibodies cross reacted with rat nectins and were used to detect endogenous rat nectins. Rat monoclonal anti-N1 antibody N1 (clone 4B12; MBL International Corporation) and anti-N3 antibody N3 (clone 103-A1; MBL International Corporation) antibodies, which recognized the EC regions of N1 and N3, respectively, were used to detect exogenously introduced mouse nectins. These monoclonal antibodies did not immunocytochemically detect rat endogenous nectins in cultured cells. Other antibodies used were mouse monoclonal anti-MAP2 antibody (clone HM-2, Sigma-Aldrich), rabbit anti-MAP2 antibody (Chemicon), mouse monoclonal anti-beta-tubulin antibody (clone P21C6; Chemicon), mouse monoclonal anti-beta-catenin antibody (clone 4G10; a gift from M.J. Wheelock, University of Nebraska, Omaha, NE), rabbit anti-beta-catenin antibody (Sigma-Aldrich), rat anti-GFP antibody (Nacalai Tesque), rabbit anti-FLAG antibody (Sigma-Aldrich), mouse monoclonal anti-N-cadherin antibody (Transduction Laboratories), and mouse antisyaptotagmin antibody (Chemicon).

**Western blotting**

Neuronal cultures were prepared in 35-mm petri dishes, and their lystate were analyzed by SDS-PAGE in which the total protein concentration had been adjusted to be equal for each lane. Proteins were transferred to a nitrocellulose membrane, the membrane was blocked with 5% skim milk for 1 h, and membranes were incubated overnight at 4°C with anti-nectin or anti-N-cadherin antibodies in Can Get Signal solution (Toyobo). Blots were washed with TBS, incubated for 1 h in HRP-conjugated goat anti-mouse antiserum (1:5,000; Jackson ImmunoResearch Laboratories), and washed with TBS, incubated overnight in 3% BSA in TBST at room temperature or 37°C. The signals on the films were digitally scanned and analyzed by using Scion Image densitometric analysis.

**Image acquisition and quantification of dendrite morphology**

Images of neurons were obtained with a confocal microscope (LSM510; Carl Zeiss MicroImaging, Inc.), equipped with a 63 × 1.4 or a 40 × NA 1.3 lens using LSM510 software (Carl Zeiss MicroImaging, Inc.), and their morphology was analyzed with the same software and with Adobe Photoshop. For quantification of the dendritic arbor pattern, the number of dendrites shorter than the diameter of neuronal soma were chosen for this measurement. The number of branches protruding from these dendrites was also manually counted. To obtain the circle-crossing index of dendritic arbor, we superimposed a circle of 40 μm in diameter on the center of the cell body of each neuron. Then, the number of dendrites crossing the circle was counted and plotted; subsequently, Welch’s t test was performed. In general, several neurons were randomly chosen from multiple culture plates for each assay. Neurons at 7–8 DIV were used for these analyses.

**Online supplemental material**

Fig. S1 shows the localization of exogenous nectins in nectin-transfected mature neurons as well as the effects of nectin overexpression on the N-cadherin level. Fig. S2 shows the effects of expression of nectin mutants on neurite patterning. Fig. S3 shows the effects of nectin overexpression on synaptotagmin distribution. Fig. S4 shows nectin and beta-catenin distribution in nectin-transfected 293 cells. Fig. S5 shows nectin and beta-catenin distribution in a mixed culture of N1- and N3-transfected 293 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200601089/DC1.

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