Highlights

- APEX proteomics extended to open cellular domains, cell surface, and primary cells

- 199 glutamatergic and 42 GABAergic synaptic cleft proteins enriched

- Ten candidates validated as synaptic proteins by imaging and/or synaptosome blotting

- Mdga2 regulates specificity of presynaptic recruitment to inhibitory post-synapses

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In Brief

An approach enabling proteomic analysis of subcellular compartments that are not membrane enclosed reveals unique features of excitatory and inhibitory synapses.
Proteomic Analysis of Unbounded Cellular Compartments: Synaptic Clefts

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http://dx.doi.org/10.1016/j.cell.2016.07.041

SUMMARY

Cellular compartments that cannot be biochemically isolated are challenging to characterize. Here we demonstrate the proteomic characterization of the synaptic clefts that exist at both excitatory and inhibitory synapses. Normal brain function relies on the careful balance of these opposing neural connections, and understanding how this balance is achieved relies on knowledge of their protein compositions. Using a spatially restricted enzymatic tagging strategy, we mapped the proteomes of two of the most common excitatory and inhibitory synaptic clefts in living neurons. These proteomes reveal dozens of synaptic candidates and assign numerous known synaptic proteins to a specific cleft type. The molecular differentiation of each cleft allowed us to identify Mdga2 as a potential specificity factor influencing Neuroligin-2’s recruitment of presynaptic neurotransmitters at inhibitory synapses.

INTRODUCTION

The mammalian brain is capable of complex cognition because individual nerve cells assemble into higher order circuits that receive, process, store, and transmit information. Central to this information flow are chemical synapses, specialized junctions between communicating neurons that mediate neurotransmitter release and recognition. Because synapses’ functions, along with their formation, remodeling, and elimination, are so central to brain function, there is tremendous interest in dissecting the molecular architecture and functional properties of synapses.

Microscopy and mass spectrometry (MS)-based proteomics have been applied extensively to study the protein composition of synapses. Though powerful in its ability to provide spatial context, microscopy is limited when specific antibodies against target proteins are not available or when recombinant tagging raises concerns about mislocalization or overexpression. Microscopy is also low throughput and more often applied to validate hypotheses than to perform an unbiased search for novel proteins.

MS-based proteomics, on the other hand, is ideally suited for high-throughput and unbiased detection of endogenous proteins. However, it sacrifices spatial information because analysis is performed after cell lysis. Fractionation schemes, such as to enrich entire synaptic terminals (i.e., synaptosomes) (Biesemann et al., 2014), synaptic vesicles (Takamori et al., 2006), the postsynaptic density (PSD) (Bayés et al., 2012), and the active zone (Boyken et al., 2013), recover some spatial information but vary greatly in their degrees of purity. For example, mitochondrial, nuclear, and glial (Fifenn et al., 1976) contaminants are common in synaptosome and PSD preparations, and key proteins are frequently lost (Figure S1). In addition, fractionation usually blends across many synapse types. Synaptosome purifications, for example, do not distinguish between excitatory glutamate-releasing synapses and inhibitory GABA-releasing synapses, whose molecular compositions are quite different, due to their antagonistic functions. Finally, a major limitation of purification-based MS approaches is that many of the subdomains of the synapse are impossible to purify and therefore inaccessible to MS proteomic analysis. This includes the synaptic cleft and the inhibitory post-synaptic region, which lacks a detergent-insoluble “density,” in contrast to the excitatory PSD.

Recently, we (Rhee et al., 2013) and others (Roux et al., 2012) have reported approaches to proteomic mapping that bypass the need for organelle or subdomain purification and instead target “promiscuous” tagging enzymes to the subcellular region of interest (APEX or BioID, respectively). In live cells, addition of a small molecule substrate triggers enzyme-catalyzed biotinylation of its neighboring endogenous proteins (Figures 1A and 1B). Subsequently, biotinylated proteins are enriched with streptavidin beads and identified by MS. The main advantages of this approach are that unpurifiable cellular regions, such as the synaptic cleft, can in principle be targeted for proteomic mapping, and the resulting data are potentially more accurate, because tagging is performed in living cells while membranes and protein complexes are still intact and artifacts resulting from detergent lysis and serial centrifugation are avoided.
Though such technology could be highly enabling for the study of synapse molecular architecture, numerous hurdles must be surmounted. First, neither APEX nor BioID have been demonstrated in neurons. Second, we have only used APEX for proteomic mapping in membrane-enclosed compartments (the mitochondrial matrix [Rhee et al., 2013] and mitochondrial intermembrane space [Hung et al., 2014]), not “open” subcellular regions such as the synaptic cleft. It is unclear what spatial specificity is achievable in such an environment. BioID is expected to have a larger labeling radius than APEX, because the half-life of its reactive intermediate, biotin-AMP, is minutes in water, in contrast to <1 ms for the biotin-phenoxyl radical generated by APEX-catalyzed oxidation. Third, synapses constitute a tiny fraction of neurons by mass. It is unclear if existing protocols can enrich a small biotinylated proteome over the much larger non-biotinylated proteome: previous APEX studies have not addressed this issue.

Figure 1. Design and Characterization of Peroxidase Fusion Constructs for Proximity Biotinylation

(A) Scheme of peroxidase-mediated proteomic tagging in the synaptic cleft. Horseradish peroxidase (HRP) is genetically targeted to the cleft via fusion to a known cleft protein. The gray shapes are endogenous proteins residing inside and outside the synapse. To initiate labeling, the membrane impermeant biotin-phenol conjugate BxP (red B, biotin; chemical structure in B) is added to the live neurons for 1 min together with the oxidant H$_2$O$_2$. HRP converts BxP into a phenoxyl radical, which covalently tags proximal endogenous proteins at electron-rich side chains such as Tyr (Rhee et al., 2013). Subsequently, neurons are lysed and biotinylated proteins are isolated using streptavidin beads for identification by mass spectrometry (MS).

(B) Structure of BxP and BP probes.

(C) HRP fusion constructs employed in this study. HRP-TM is a general cell-surface construct.

(D) Fluorescence imaging of synaptic HRP fusion constructs with respect to excitatory and inhibitory synapse markers, vGlut1 and vGAT. For maximum detection sensitivity, the HRP constructs were visualized via BxP labeling followed by neutravidin-AlexaFluor647 staining (red). Scale bars, 10 µm. See also Figures S1–S4 for additional characterization of constructs and their expression levels.

(E) Quantitation of colocalization extent for images in (D) plus seven other fields of view containing >900 puncta per construct. Errors, ± 1 SD

(F) Electron microscopy (EM) of HRP fusion constructs. HRP catalyzes the polymerization and local deposition of diaminobenzidine, which recruits electron-dense osmium (Martell et al., 2012). SV, synaptic vesicles. Scale bars, 200 nm. See also Figure S2C for additional EM.
targeted much more abundant structures, such as mitochondria. Fourth, it is challenging to localize APEX activity specifically to synaptic subdomains of interest. Though cleft-resident proteins are known, all of these also have pools elsewhere in the neuron, such as the secretory pathway. How can we achieve specific proteomic tagging only in the cleft and avoid capturing the endoplasmic reticulum (ER) or Golgi proteomes as well?

Here, we describe modifications to the APEX technique to enable successful proteomic mapping of the neuronal synaptic cleft. To begin to probe the vast diversity of synapses in the brain, we generate two independent proteomic lists: one representative of excitatory, glutamate-releasing synapses and one representative of inhibitory, GABA-releasing synapses. We analyze these proteomes to show that specificity and depth of coverage are both higher than for previous proteomes obtained by biochemical fractionation. Follow-up imaging and western blotting provide synapse validation for ten proteomic hits. Finally, we perform functional studies in neuron culture on two post-synaptic membrane proteins identified in our proteomes, Mdga1 and Mdga2, and uncover differences in their regulatory effects on Nlgn2, suggesting potential roles in setting synapse specificity.

**RESULTS**

**Establishing a Peroxidase-Based Platform for Proteomic Mapping of the Synaptic Cleft**

Though APEX tagging has been performed in *Drosophila* larval muscle (Chen et al., 2015), reagent delivery to tissue was a concern, in addition to *H₂O₂* toxicity. We therefore opted to use dissociated neuron culture, rather than intact brain tissue, to allow for rapid delivery and washout of biotin-phenol, *H₂O₂*, and subsequent peroxidase quenchers. At DIV19 (19 days in vitro), our embryonic rat cortical neuron cultures displayed abundant staining of synapse markers (Bassoon, vGlut1, and vGAT), and synapses looked normal by electron microscopy (EM) (Figures 1F and S2C).

When designing the peroxidase fusion constructs to use for proteomic mapping in the synaptic cleft, our first concern was to maximize the activity of the peroxidase, because the proteomic region of interest is so small. APEX2 is the second-generation, more active variant of APEX (Lam et al., 2015). However, the commonly used horseradish peroxidase (HRP) enzyme is even more active than APEX2. For applications in the cytosol, nucleus, and mitochondria, HRP cannot be used, because its four structurally essential disulfide bonds do not form in reducing environments, leaving HRP inactive (Martell et al., 2012). However, HRP is active in the oxidizing secretory pathway and cell surface and catalyzes the same labeling chemistry as APEX2 with biotin-phenol![](https://cell.com/journals/figs/F183CB592F3AC378A447A0F9254D17F7.png)

We therefore generated N-terminal, extracellular-fusing fusions of HRP with three known glutamatergic excitatory synaptic cleft-resident proteins (Nlgn1, Lrrtm1, and Lrrtm2 [Linhoff et al., 2009; Song et al., 1999]) and two known GABAergic inhibitory synaptic cleft resident proteins (Slitrk3 and Nlgn2 [Chih et al., 2006; Takahashi et al., 2012; Varoqueaux et al., 2004]) (Figure 1C). Surprisingly, HRP-Nlgn1 exhibited poor specificity for excitatory over inhibitory synapses, by imaging and in a preliminary MS proteomic experiment (Figure S1), and was not characterized further. The remaining four constructs were highly enriched at synapses (Figures S2A and S2B), gave the expected preference for excitatory versus inhibitory synapses (Figure 1D and 1E), and showed specific and robust HRP activity in the cleft by EM (Figures 1F and S2C). Using lentiviral transduction, we titrated down expression levels to 40%–70% that of the endogenous protein or mRNA counterpart (Figures S3A–S3C). Under these expression conditions, we did not observe changes in synapse size or density in transduced neurons (Figures S3E and S3F).

As expected, treatment with biotin-phenol (BP) and *H₂O₂* gave labeling of both cell-surface and intracellular proteins, likely in the secretory pathway proximal to ER and Golgi pools of the HRP fused proteins. We reasoned that we could improve specificity for the extracellular cleft proteome by rendering BP membrane impermeant. We synthesized a variant, called BxpP, with a long and polar polyamide linker (Figure 1B). Figure 2A shows that BxpP still gives robust biotinylation with HRP and *H₂O₂* but no longer enters cells (no signal observed with intracellular constructs PSD95-APEX2 and APEX2-NE). When applied to the synaptic HRP fusion constructs, BxpP produced HRP- and *H₂O₂*-dependent biotinylation on the neuron surface that was punctate in appearance (Figures 1D, S4A, and S4B). By contrast, HRP-TM, a control construct that expresses over the entire neuron surface, produced a diffuse rather than punctate BxpP labeling pattern.

We ran biotinylated neuron lysates on gel and analyzed them by streptavidin blotting. Figures S4C and S4D show that each HRP fusion construct biotinylates a wide range of endogenous proteins, in an *H₂O₂*-dependent manner. However, when we proceeded to enrich these biotinylated proteins using streptavidin-coated beads according to our previous protocol, we found that endogenous cytosolic proteins such as PSD95, which should not be tagged by HRP and BxpP, were also enriched (Figures 2C and 2D). We hypothesized that our previous enrichment protocol was disassembling the detergent-insoluble and tightly crosslinked post-synaptic density (PSD), which was coming down with the biotinylated cleft proteome. We therefore modified the protocol by adding a 1% SDS lysis step with 10 min boiling to disrupt the PSD and found that this removed undesired cytosolic proteins while preserving enrichment of desired synaptic surface proteins such as GluA1 (Figure 2D). Figure 2B shows streptavidin blot and silver stain visualization of our enriched biotinylated proteomes.

**Proteomic Mapping of Excitatory Glutamatergic and Inhibitory GABAergic Synaptic Clefts**

Previously, we found that a “ratiometric” APEX-tagging strategy improved the specificity of protein identifications in the mitochondrial intermembrane space (IMS), a compartment that is leaky to biotin phenoxyl radicals, due to porins in the outer mitochondrial membrane ([Hung et al., 2016](https://www.cell.com/journals/figs/2151-0004.png)). In the ratiometric approach, for each detected protein, we compare its extent of biotinylation by targeted peroxidase (e.g., synaptic HRP fusion construct) versus non-targeted peroxidase (e.g., HRP-TM, which targets HRP evenly over the entire neuron surface). If a protein is biotinylated more extensively by synaptic HRP than by HRP-TM, we retain it for our proteome. If it is biotinylated...
more extensively by HRP-TM than by synaptic HRP, we reject it: such proteins may be just outside of synapses, accessible to the biotin radical, but not actually a synaptic protein.

Because the synaptic cleft is open and non-membrane-enclosed, it represents a greater challenge than the mitochondrial IMS in terms of spatial specificity. We reasoned that it would be essential to use the ratiometric tagging approach. We also wanted to capitalize on the availability of two validated HRP fusion constructs for each synaptic cleft type—Lrrtm1 and Lrrtm2 for the excitatory glutamatergic cleft and Slitrk3 and Nlgn2 for the inhibitory GABAergic cleft. An endogenous protein enriched by two different excitatory HRP constructs is much more likely to be a true positive identification than a protein enriched by only one. With these considerations in mind, we designed the three independent proteomic experiments shown in Figure 3A. Each experiment combines four cellular samples: one biotinylated by an excitatory HRP fusion construct; one biotinylated by an inhibitory HRP fusion construct; one biotinylated by HRP-TM; and one non-biotinylated negative control. The four samples are then combined and analyzed as a pooled mixture by liquid chromatography and tandem MS/MS.

As shown in Figure 3B, each experiment identified >2400 unique proteins. The vast majority of these were non-specific streptavidin bead binders, identifiable by their low 114/117 or 115/117 iTRAQ ratios. After removing these proteins (“Filter 1”, Figure 3C), we used the 114/116 and 115/116 iTRAQ ratios to identify proteins preferentially enriched by synaptic HRP constructs over the HRP-TM control construct (“Filter 2”, Figure 3D). Then, the three independent excitatory synaptic cleft datasets were intersected, as well as the three independent inhibitory synaptic cleft datasets. Figures S5B and S5C illustrate the importance of the three-way intersection to improve the quality of each proteomic list. The last filter (“Filter 3”, Figure 3E) was based on excitatory/inhibitory biotinylation ratio: we removed from the excitatory proteome the small number of proteins that were much more strongly biotinylated by inhibitory HRP constructs than excitatory HRP constructs, and vice versa. After these filtering steps, our final excitatory and inhibitory proteomic lists consisted of 199 (Table S1, Tab 1) and 42 (Table S1, Tab 2) unique proteins, respectively.

**Characteristics of the Two Proteomic Lists**

As expected, each proteomic list contains numerous ion channels, GPCRs, adhesion proteins, and transporters (Figure 4A).
Both post-synaptic membrane proteins and pre-synaptic membrane proteins are represented, in addition to soluble secreted proteins such as Nptx1 (Figure 4B). We also observe some secreted proteins that may be of glial origin, such as Gpc6, which promotes excitatory synapse formation (Allen et al., 2012). Our excitatory synapse cleft list contains all four AMPA receptor subunits and three NMDA receptor subunits, while seven GABAΔ receptor subunits appear in the inhibitory synaptic cleft list. The overlap between the two proteomic lists is 20 proteins (Table S1, Tab 3), which includes known dual-localized synaptic proteins such as Erbb4 (Krivoshheyev et al., 2008), Grik2 (Lerma, 2003), and Gabbr1 (Kulik et al., 2002).

To characterize the specificity of each proteomic list, we first checked for intracellular cytosolic proteins. Both lists lack any protein known to be intracellular (Table S1, Tabs 1 and 2, Column AN), attesting to the effectiveness of BxxP in restricting biotinylation to the cell surface. We then determined synapse specificity by calculating the fraction of each proteome with prior literature connection to synapses. 84% of the excitatory proteome and 90% of the inhibitory proteome have previous synapse annotation (Figure 4B, left). The remaining 33 proteins (29 from the excitatory proteome, 2 from the inhibitory proteome, and 2 in both; Table S2) that lack synapse annotation, which we call "synapse orphans," could be false positives, or they could be newly discovered synaptic proteins. Below, we present imaging and western blotting data on ten of these synapse orphans, supporting the latter possibility.

To determine the excitatory versus inhibitory synapse specificity of each proteomic list, we analyzed the subset of proteins in each list with known excitatory or inhibitory synapse localization (Figure 4B, middle). The excitatory proteome is highly enriched for proteins known to reside at excitatory synapses or at both synapse types (98%). The inhibitory proteome is enriched for known inhibitory synapse proteins (62%), but also includes a significant number of proteins with excitatory annotation. Because the inhibitory synapse is poorly characterized and the literature is biased toward assays of excitatory synapse localization, it is possible that many of these proteins are actually dual localized to both excitatory and inhibitory synapses. Indeed, follow-up experiments described below and shown in Figure S6 suggest dual localization for four excitatory-annotated proteins that appear in our inhibitory synaptic cleft proteome.

Figure 4C provides an illustration of the synapse subtype specificity of both proteomic lists, showing for example that AMPA and NMDA receptor subunits are detected only in the excitatory proteome, while GABAΔ receptor components are detected exclusively in the inhibitory proteome. Interestingly, previous studies have suggested that some inhibitory synapse components “leak over” to excitatory synapses, and vice versa, perhaps to enable cross-talk or regulation between synapse types (Chen et al., 2012). Consistent with these studies, our data show that the inhibitory GABAΔ receptor subunit Gabbr1 resides at excitatory synapses as well (Kulik et al., 2002) and the excitatory kainate receptor Grik2 can also be found at inhibitory synapses (Lerma, 2003).

Due to our experimental design, every protein appearing in our lists is associated with an E/I (excitatory/inhibitory) ratio, based on the 114/115 iTRAQ ratio, that reflects its enrichment at excitatory versus inhibitory synapses. This can be visualized in the scatterplots shown in Figures 4D and 5D. Known excitatory synapse-specific proteins such as AMPA receptors (Gria2-3) and NMDA receptors (Grin1) appear below the
diagonal, whereas known inhibitory synapse-specific proteins such as GABA_A receptors (Gabra1,3, Gabrb1-3) lie above the diagonal. Dual-localized proteins such as Erbb4 are close to the diagonal.

To characterize the sensitivity, or depth of coverage, of each proteome, we generated separate lists of well-established excitatory cleft-resident or inhibitory cleft-resident proteins (Table S3). Of the 62 such excitatory proteins, our proteome contained 43 (69%). Of the 24 inhibitory proteins, we enriched 11 (46%). The proteins we missed may be sterically shielded from biotinylation through protein or membrane interactions in the live-cell context. Alternatively, they could be dual localized, with both a synaptic population and a non-synaptic population, and consequently removed by our Filter 2 step (Figures 3B and 3D), which considers the ratio of biotinylation by synapse-localized HRP versus general cell-surface HRP-TM. Finally, some genes may not be expressed in the specific rat cortical neuron preparations that we used.
Proteomes Reveal New Synaptic Proteins

Within each proteome, ~85% of proteins have prior literature connection to synapses. The remaining ~15% are “synapse orphans,” with no previous literature assigning them to synapses. We found 29 such orphans in the excitatory cleft, 2 in the inhibitory cleft, and 2 in both clefts (some examples in blue font in Figure 4A; complete list of orphans in Table S2, Tab 1). Guided by the availability of commercial antibodies and transgenes for recombinant expression, we selected 14 excitatory synapse orphans for follow-up analysis (Figure 5A).

To perform fluorescence imaging, we expressed 7 recombinant orphan proteins in DIV19 neuron cultures, via lentiviral transduction. We found that extremely low expression levels were essential to observe punctate rather than diffuse localization patterns, perhaps because mistargeting occurs upon overexpression. However, GFP and V5 epitope tags were not visible at such low expression levels; instead, we used HRP in combination with BxxP as a highly sensitive, amplifying imaging label (Figure S6 shows the superior sensitivity of HRP over Venus for fluorescence imaging). Figures 5B and 5D show that four orphan proteins from the excitatory proteome, tagged with HRP at their N-terminal ends, colocalize with endogenous Bassoon as well as vGlut1, a marker of glutamatergic synapses.

For eight synapse orphans, we obtained commercial antibodies and used these to probe for the endogenous proteins in purified synaptosomes derived from adult rat brain. A concern when using neuron cultures is that synapses could form between neurons that do not normally contact each other in vivo. By contrast, our synaptosomes are derived from physiological synapses already present in the adult rat brain. For seven orphans, we observed clear enrichment of the endogenous protein in synaptosome fractions compared to non-synaptosome
fractions such as S2 (Figures 5E and 5F). The eighth antibody, against Notch3, failed to detect Notch3, even in whole neuron lysate (data not shown). Thus, for seven synapse orphans, the combination of enrichment at synaptic clefts in live neuron culture (via HRP tagging) and enrichment in synaptosomes derived from adult brain tissue provides strong and orthogonal evidence that these proteins are bona fide synaptic proteins.

One of these antibodies, against Notch2, also worked for visualization of the endogenous protein, both in neuron culture and in adult rat brain tissue. Figures 5G and 5H show colocalization of Notch2 and the synapse marker Bassoon, providing a third line of evidence that Notch2 is synthetically localized.

Altogether, of the 14 synapse orphans we analyzed by imaging and immunoblotting, positive identifications were made for 10 of them (Figure 5E). For the remaining four, two were inconclusive (non-specific antibody for Notch3 and HRP tag disruption of surface trafficking for Matn2). Negative results were obtained for HRP-tagged Brinp2 and Sm2p33b, but we suspect that these soluble, secreted proteins may be especially sensitive to HRP tagging and could mislocalize. This is supported by the observation that HRP-tagged Brinp3 (homologous to Brinp2) also appeared non-synaptic by imaging, but due to availability of a specific antibody for this protein, we were able to assign endogenous Brinp3 to synapses via synaptosome immunoblotting (Figure 5E). Therefore, our statistics, albeit on a small sample size, suggest that our synaptic cleft proteomic lists have very low false-positive rates (i.e., the 84%-90% synapse specificity represents a lower bound) and may be a rich source of synapse protein candidates.

The proteomic datasets can also be mined for insights on the synapse sub-type specificity of known synaptic proteins. For example, for the 38 known synaptic proteins in our inhibitory synaptic cleft list, 17 were not previously known to reside at inhibitory GABAergic synapses specifically. Interestingly, the proteomic lists also highlight 11 proteins previously annotated as excitatory, which we detected at both excitatory and inhibitory synapses in our proteomic datasets. To follow up on four of these proteins, we performed fluorescence microscopy with HRP-tagged recombinant proteins introduced by lentiviral transduction. Figure 5F shows that all four of these proteins (Flrt2, EphB6, Dcc, and Elfn1) significantly colocalize with both vGlut1 and vGAT markers, suggesting that they reside at both synaptic types, at least in neuron culture.

**CD200 Is an Excitatory Synapse-Localized Protein that Regulates Synapse Numbers in the Visual Thalamus**

For many of the proteins we enriched, the only prior evidence for synaptic localization was enrichment in a synaptosome MS study. As described above, the high false-positive rates of such studies necessitate extensive follow-up experimentation to distinguish true positives from false positives. By contrast, our lists are much more specific. Therefore, if a protein is detected in a synaptosome preparation and in our live-cell proteomic map, it is much more likely to be a genuine synaptic protein. CD200 is one of the most highly enriched proteins in our excitatory synaptic cleft proteome. CD200 was previously identified in a synaptosome MS study (Biesemann et al., 2014) (which is why we did not classify it as a synapse orphan), but there has been no further characterization of this protein in neurons. We performed immunostaining of CD200 in brain tissue from wild-type mice and found that it was localized throughout the neuropil, as expected for an excitatory synaptic protein (Figure 6F). In the visual thalamus, CD200 expression was highest during early postnatal development (P10), which could indicate a role in synapse development or remodeling. We then used structured illumination microscopy (SIM) to assess whether CD200 colocalized with synaptic markers in vivo. Indeed, CD200 colocalized with both presynaptic marker vGlut2 and postsynaptic marker Homer in the P10 dorsal lateral geniculate nucleus of the thalamus (dLGN) (Figure S6G).

To probe the functional role of CD200 at synapses, we used high-resolution confocal microscopy to quantify the number of colocalized vGlut2 and Homer puncta in the dLGN of CD200 knockout (KO) mice at P10 (Figures S6H and S6I). We observed a significant reduction in synapse numbers compared to wild-type littermate controls, demonstrating that the function of CD200 is indeed relevant to the synapse. Future studies will be needed to determine whether CD200 plays a role in synapse formation or regulates an aspect of the synaptic refinement process.

**Mdg1 and Mdg2 Have Distinct Localizations and Regulatory Functions**

The availability of distinct proteomic datasets for the excitatory glutamatergic and inhibitory GABAergic synapses provides an opportunity to consider the complement of molecules that define and contribute to the specific formation of each synapse type. For example, specific synaptic adhesion proteins are known to play important roles in recruiting GABA vesicle-containing pre-synapses to GABA receptor-containing post-synaptic membranes and glutamate vesicle-containing pre-synapses to glutamate receptor-containing post-synaptic membranes.

Our attention was drawn to two proteins, Mdg1 and Mdg2, that have been linked in previous studies to the well-studied adhesion protein Nlg2 (Lee et al., 2013) and Pettem et al. (2013) have shown that both Mdgas bind to Nlg2 and that Mdg1 acts via Nlg2 to downregulate inhibitory synapse formation. Due to high (~70%) sequence homology, Mdg2 was assumed to have the same localization and function as Mdg1, though Mdg2 has not previously been studied in neurons. Interestingly, we detected Mdg1 in our excitatory synaptic cleft proteome and Mdg2 in our inhibitory proteome, which challenges this assumption and suggests that Mdg2 may have a different function at synapses than Mdg1.

To further investigate Mdg1 and Mdg2, we prepared recombinant fusions to HRP, since specific antibodies are not available, and imaged the proteins in DIV19 neurons. In agreement with the proteomic data, recombinant Mdg2 overlapped with the inhibitory marker vGAT exclusively (Figure 6A). Recombinant Mdg1, on the other hand, overlapped with both excitatory and inhibitory markers (Figure S7A). The discrepancy between this observation and our proteomic data could result from the incomplete coverage of the inhibitory proteome, which recalled only 46% of expected proteins. Previous imaging of GFP-Mdg1 also showed overlap with both excitatory and inhibitory terminals (Pettem et al., 2013). Thus, Mdg1 is most likely localized to both...
synapse types, while Mdg2 is specifically localized to GABAergic synapses only.

We sought to investigate the hypothesis that Mdg2 has a different function at synapses than Mdg1. Our assay capitalizes on the synaptogenic activity of Nlgn2 (Graf et al., 2004), which when overexpressed on the post-synaptic membrane, over-recruits both vGlut1- and vGAT-positive pre-synaptic terminals (Takahashi et al., 2012) (Figure S7B). When Mdg1 was co-overexpressed with Nlgn2, the enhanced recruitment of both vesicle types was suppressed (Figures 6B and 6C). Co-overexpression of Mdg2, however, suppressed selectively the recruitment of excitatory but not inhibitory vesicles.

Based on these observations, we hypothesized that Mdg1 and 2 both downregulate the trans-synaptic vesicle recruiting activity of Nlgn2 (which occurs via unknown presynaptic binding partner[s]) (Figure S7C). However, Mdg1 binds Nlgn2 in such a way that it blocks recruitment of both inhibitory and excitatory vesicles. MDGA2 binds Nlgn2 differently, blocking recruitment of only excitatory vesicles while allowing recruitment of inhibitory vesicles.

To further test this hypothesis, we performed shRNA knockdown of Mdg1, Mdg2, or both together (Figures 6E, 6F, and S7D). The interpretation of the data requires the assumption that each Nlgn2 molecule binds to Mdg1 or 2, but not to both at once. In the case of Mdg1 knockdown, more Nlgn2 is freed to interact with Mdg2 instead. According to our model, Mdg2 promotes inhibitory vesicle recruitment, but not excitatory. Correspondingly, we observe that Mdg1 knockdown causes an increase in inhibitory vesicle signal, but not excitatory signal. This is also consistent with previous observations (Lee et al., 2013; Pettem et al., 2013).

By itself, Mdg2 knockdown had no significant effect, but when combined with Mdg1 knockdown caused both inhibitory and excitatory vesicle signals to increase, consistent with full derepression of Nlgn2 activity. Because this phenotype is distinct from that caused by knockdown of Mdg1 only (increase...
in inhibitory vesicle density only), it suggests that Mdgα2 plays a role in downregulation of excitatory vesicle recruitment.

We also performed a gain-of-function assay by overexpressing only Mdgα1 or Mdgα2 (without Nlg2 co-overexpression) (Figure 6D). More Mdgα2 in neurons might shift the equilibrium for Nlg2, causing more of it to bind to Mdgα2 than Mdgα1. Accordingly we would expect to see increased inhibitory vesicle recruitment, with no effect on excitatory vesicle recruitment. Our data in Figure 6D shows this expected trend.

Fluorescence imaging of a panel of Mdgα1/2 chimeras (Figure 6G) showed that their extracellular juxtamembrane Ig4-6 regions are responsible for their unique synaptic localizations (Figures 6H and S7E). Chimeras with the Ig4-6 region from Mdgα1 exhibited “Mdgα1-like” localization to both excitatory and inhibitory synapses, while chimeras with the Ig4-6 domain from Mdgα2 exhibited “Mdgα2-like” localization to inhibitory synapses only. Previous studies have shown that a different region of the Mdgas, the Ig1-3 domain, mediates cis-interactions with Nlg2 (Lee et al., 2013; Pettem et al., 2013). Therefore, we postulate that the Mdgas target to excitatory and/or inhibitory synapses, governed by their Ig4-6 regions, and independent of interactions with Nlg2. At inhibitory synapses, each Mdgα then interacts with Nlg2 to differentially regulate its activity. At excitatory synapses, perhaps the role of Mdgα1 is to help prevent the invasion of inhibitory synaptic elements (such as pools of Nlg2 itself) into excitatory terminals.

**DISCUSSION**

In this study, we develop and extend the APEX platform to achieve successful proteomic mapping of the synaptic cleft. We replace APEX2 with HRP, which is more active at the cell surface, and introduce the BxXP probe for restriction of peroxidase labeling to the neuronal cell surface. We apply an intersectional strategy, using two independent peroxidase fusion constructs targeting the same cellular locale, in order to dramatically improve the specificity of protein identifications. To adapt the technique to primary, non-dividing cells, we employ post-digestion iTRAQ chemical labeling for quantitation, rather than SILAC metabolic labeling, which requires protein turnover. Finally, to overcome background caused by the unique, detergent-insoluble matrix underlying the post-synaptic membrane, we develop a denaturing lysis and streptavidin enrichment procedure that effectively separates intracellular proteins from cleft-exposed transmembrane proteins.

Our study opens the door for APEX to be applied to a greater diversity of cellular structures. This includes other unpurifiable subdomains of the synapse (e.g., the inhibitory post-synaptic region and synaptic ribbons), as well as smaller and more challenging domains across cell biology in general, for example, mitochondria-ER contact sites, RNA granules, the axon initial segment, and even macromolecular complexes. Because peroxidase-catalyzed proteomic tagging occurs in just 1 min (in contrast to BioID which requires 18–24 hr of labeling), it should also be possible to map proteomes under different cell states, such as in response to drugs or LTP, at different synapse maturities, or in models of brain disease.

The excitatory and inhibitory synaptic cleft proteomic lists generated by this study depart from existing synapse proteomes in several respects. First, they are much more specific. Due to contamination by mitochondrial, nuclear (Figures S1A and S1B), and glial proteins, synaptosome and PSD preparations typically have false discovery rates of 20%–40% (Biesemann et al., 2014). This necessitates extensive follow-up experimentation to distinguish genuine synaptic proteins from false positives. By contrast, our datasets have false discovery rates of <10%, or likely much lower, based on our analysis of synapse orphans (Figure 5). Second, our lists have higher coverage (Figure S1C). By tagging in live cells and by-passing detergent lysis and serial centrifugation, we improve protein recovery, enabling us to identify dozens of proteins that were missed by previous synaptosome, PSD, and active zone preparations. These include soluble, secreted proteins such as Gpc6 and Reelin that may dissociate from organelles during centrifugation. Third, our lists chart an important subdomain of the synapse that has eluded previous MS studies because it cannot be purified. Fourth, our lists cleanly separate components of the glutamatergic synapse from components of the GABAergic synapse, in contrast to synaptosome preparations, which blend across all synapse types, and PSD preparations, which are applicable only to excitatory synapses because inhibitory synapses lack a PSD. Biesemann et al. (2014) have attempted to further purify synaptosomes by FACS, but their resulting vGlut1-enriched dataset is imperfect, containing inhibitory synapse components such as Gabgr2, Gabra1, Gabra5, and gephyrin. Here, using the power of genetic targeting, we achieve >98% specificity for excitatory components in our glutamatergic list and >76% specificity for inhibitory components in our GABAergic list (value is corrected based on data shown in Figure S6).

Our inhibitory synapse proteome can also be compared to two previous studies that use immunoprecipitation MS, rather than biochemical fractionation, to identify components of the GABAergic synapse (Heller et al., 2012; Kang et al., 2014). Though the Heller et al. study that uses GABA_A receptor α1 immunoprecipitation is quite specific, both datasets miss the majority of known inhibitory synaptic cleft components (coverage <34%), probably because the baits do not interact directly or stably with these proteins. By contrast, our mapping approach does not require direct interactions, because the biotin-phenoxyl radical diffuses out of the peroxidase active site to tag endogenous proteins in the neighborhood of HRP/APEX.

Both synaptic cleft proteomic lists can be mined for biological insights or hypotheses. We have illustrated this by using our datasets to discover ten synaptic cleft proteins (validated by microscopy and/or synaptosome immunoblotting in Figure 5) and reveal a potential inhibitory synapse component for four known excitatory synaptic cleft proteins (Figure S6). We also followed up on CD200, a protein previously linked to synapses only by crude synaptosome MS data. After observing strong enrichment in our excitatory proteome, we found that CD200 is highly expressed in the visual thalamus during periods of synaptic refinement, and its deletion perturbs the normal development of retinogeniculate synapses.
By also revealing different synaptic sub-type localizations for two known, homologous synaptic proteins (Mdga1 and Mdga2), our proteomic data inspired the hypothesis that these two proteins have different functional roles. Follow-up experiments using gain-of-function and loss-of-function assays in neuron culture suggest that while Mdga1 may act to generally downregulate Nlgn2 activity, Mdga2 may function as a specificity factor at inhibitory synapses to downregulate Nlgn2’s signaling with vGlut1 pre-synaptic terminals, but not vGAT pre-synaptic terminals.

There are many more synapse orphans and synapse subtype orphans identified in our study, on which we did not perform follow-up experimentation. Several of these are intriguing and, if validated, could open up avenues for exploration. For example, Csmd1 is a synapse orphan identified in our inhibitory synaptic cleft proteome. Though no literature describes Csmd1 as a synaptic protein, the CSMD1 gene has been linked by GWAS studies to schizophrenia (Hävik et al., 2011), and the protein may be part of the complement pathway that facilitates synaptic pruning (Hong et al., 2016; Kraus et al., 2006). The detection of endogenous Csmd1 in the inhibitory cleft of live neurons suggests a possible link between inhibitory synapse elimination and schizophrenia that could be explored in future studies. We note that Csmd1 is a very difficult protein to study by conventional techniques, because there are no specific antibodies and recombinant expression via standard techniques is not possible because the protein is enormous (~380 kDa). Proximity biotinylation and MS may be one of the only ways to investigate the subcellular localization of this protein. Interestingly, Csmd1 has been missed in all previous synaptosome, PSD, and active zone purifications.

In conclusion, our study departs from both classical synaptosome and PSD purifications, as well as previous APEX and BioID publications, and demonstrates that peroxidase-based proteomic mapping is a powerful technology for parsing the molecular properties of important nanoscale structures in biology. Mapped with few-nanometer spatial resolution and 1-min temporal resolution, the excitatory and inhibitory synaptic cleft proteomes reported here can serve as rich resources for neuroscientists.

STAR*METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.07.041.

AUTHOR CONTRIBUTIONS

K.H.L. performed all experiments except those noted below. K.H.L. and P.S.S. performed shRNA knockdowns. K.H.L. and A.S.D. performed synaptosome purifications, qPCR, and Notch2 imaging in brain slice. N.D.U., T.S., and M.H.E. processed streptavidin-enriched proteomic material and performed mass spectrometry. P.S.S., T.J.D., M.H.E., and M.H.E. performed electron microscopy. E.K.L., D.K.W., and B.S. performed CD200 experiments. K.H.L. and A.Y.T. conceived the study, designed experiments, and analyzed data. K.H.L. and A.Y.T. wrote and revised the manuscript.

ACKNOWLEDGMENTS

We thank N. Watson (Whitehead Institute Keck Microscopy Facility) for performing electron microscopy, J. Einstein for neuron cultures, A. Vignery (Yale) and J. Sedgwick (Schering-Plough) for CD200 knockout mice, and members of A. Ting’s lab, especially V. Hung, K. Cox, S. Lam, and K. Pedram, for assistance with data analysis and manuscript editing. K. Tye and G. Matthews assisted with brain slice imaging. J. Sanes, K. Shen, and S. Slavoff provided experimental advice and feedback on the manuscript, and T. Hashimoto helped with data analysis. Funding was provided by the U.S. NIH (R01-CA186568 to A.Y.T.; P41GM103412 and R01GM086197 to M.H.E.; R01-NS071008-01A1 and R01-NS092578 to B.S.) and the Howard Hughes Medical Institute Collaborative Initiative Award (A.Y.T. and S.A.C.) P.S.S. was supported by a Simons Center for the Social Brain Postdoctoral Fellowship and Feodor Lynen Research Fellowship from the Alexander von Humboldt Foundation. The Massachusetts Institute of Technology has filed a patent covering the peroxidase-based proteomic mapping technology. The authors will deposit the genetic constructs used in this work with Addgene (www.addgene.org/).


Supplemental Figures

Figure S1. Comparison of Proteomes Derived from Biochemical Fractionation versus Peroxidase Tagging, and HRP-Nlgn1 Is Not Cleanly Localized to Excitatory Synapses, Related to Figures 1 and 4

(A) Fraction of each proteome with potential mitochondrial contamination. PSDs refers to the PSD proteome obtained by Bayés et al., 2012. Syns. refers to the synaptosome study by Biesemann et al., 2014. Excit. and Inhib. are our excitatory and inhibitory synaptic cleft proteomes. Mitochondrial versus non-mitochondrial is defined by annotation in GOCC. Note that the 7 proteins with mitochondrial annotation in our proteomes also have cell surface and synapse annotation (Nptx1, Prnp, Epha4, Brinp3, Bsg; and Erbb4 in both lists).

(B) Fraction of each proteome with potential nuclear contamination. Here, nuclear is defined by annotation in GOCC, but proteins that also have synapse annotation in GOCC are omitted. All 13 such proteins enriched in our datasets are known surface-localized proteins but may have a nuclear component as well, such as Notch2.

(C) Coverage of each proteome. Fraction of a true positive list of 78 established synaptic cleft proteins detected in each dataset. Cleft proteome refers to the combination of our Excit. and Inhib. lists. See also Table S3, Tabs 1-2 for details.

(D) Fluorescence microscopy of HRP-NLGN1 as in Figure 1D with vGlut1 and vGAT markers shows that this construct localizes to both excitatory and inhibitory synapses. Quantitation shown to the right of each image, calculated from 8 fields of view (containing > 900 puncta) per condition. Errors, ± 1 s.d. Scale bar, 10 um.

(E) Preliminary proteomic mapping experiment with HRP-NLGN1. Construct was introduced by lentivirus into DIV15 cortical neurons and subsequently labeled with BxxP at DIV19. The top 50 most enriched (by 115/116 iTRAQ ratio) cell-surface proteins (intracellular proteins have been removed, as this proteomic experiment was performed prior to development of the PSD-solubilizing protocol shown in Figure 2D) contain a /C24:1 mixture of excitatory synapse-annotated proteins (green) and inhibitory synapse-annotated proteins (red), which reflects HRP-NLGN1’s excitatory/inhibitory localization ratio shown in (D).
Figure S2. Further Characterization of HRP Fusion Constructs Used for Proteomic Tagging, Related to Figure 1

(A) Fluorescence imaging of HRP fusions with respect to Bassoon, an endogenous pre-synaptic marker. Dissociated rat cortical neurons at DIV19 expressing the indicated HRP fusion construct (introduced by lentiviral transduction as in Figure 1D) were labeled live with BxxP for 1 min, fixed, and stained with neutravidin-AlexaFluor647 (green) and anti-Bassoon antibody (AlexaFluor488 readout, red). Scale bars, 10 μm.

(B) Quantitation of data in (A) along with > 10 additional fields of view per sample. Errors, ± 1 SEM.

(C) Electron microscopy of HRP fusion constructs. Same as Figure 1F but zoomed out to show multiple synapses per field of view. Dark stain indicating the presence of HRP is restricted to cleft regions and not observed on dendritic or axonal surfaces outside of synapses. Images shown are representative of > 6 images per construct. Scale bars, 400 nm.
Figure S3. Expression Levels of Hrp Fusion Constructs Used for Proteomic Tagging, Related to Figure 1

(A) Western blot analysis of HRP-Nlg2 protein expression. HRP-Nlg2 was introduced by lentiviral transduction to DIV19 dissociated rat cortical neurons as in the MS proteomic experiment. Neuron lysates were run on SDS-PAGE and blotted with anti-Nlg2 antibody. Lane 1 is the experiment, and lane 2 shows an uninfected negative control sample. Densitometry indicates that HRP-Nlg2 was present at 39% of the level of endogenous Nlg2. Accounting for the transduction efficiency of 82% (D), this corresponds to 1.5-fold overexpression in each transduced neuron.

(B) Validation of anti-Nlg2 antibody by detection of recombinant Nlg2 in HEK.

(legend continued on next page)
(C) qPCR analysis of mRNA levels with and without lentiviral transduction of HRP fusion constructs as in (A). qPCR primers were designed to amplify both the recombinant and endogenous genes. After correction for transduction efficiencies (D), the HRP constructs increase total mRNA levels by 1.4-1.7-fold in each neuron. Three technical replicates per construct; errors, ±1 SEM.

(D) Determination of transduction efficiencies by anti-V5 staining of HRP fusion constructs and anti-NeuN staining of all neuron nuclei. Neurons were prepared and infected as in (A) and (C). Scale bar, 50 μm.

(E) Calculation of relative synapse size (top) and relative synapse density (bottom) in neurons with and without lentiviral introduction of HRP fusion constructs. Samples were prepared as in (A) and (C) with the following modifications: 1.3-fold more lentivirus was used in each case to achieve 97%–99% infection efficiency (data not shown). At DIV17, a GFP marker was introduced to a sparse subset of neurons (<5%) by lipofectamine transfection. After fixation, neurons were stained for endogenous vGlut1 and vGAT as in Figure 1D. Synapse size was calculated as the area of vGlut1 or vGAT puncta overlapping with the GFP marker. >500 puncta were analyzed per sample. Synapse density was calculated by first creating a mask based on GFP. We divided the total vGlut1 or vGAT intensity in that mask by the area of the mask; this was repeated for >13 fields of view per sample. Errors, ±1 SEM.

(F) Representative images of neurons quantified in (E). Scale bar, 10 μm.
Figure S4. Characterizing the Biotinylation Activities of Synaptic HRP Fusion Constructs, Related to Figures 1 and 2

(A) Fluorescence imaging of the biotinylation catalyzed by HRP fusion constructs used for proteomics. DIV19 rat cortical neurons expressing the constructs indicated across the top were treated with BxxP and H\textsubscript{2}O\textsubscript{2} for 1 min, then fixed and stained with neutravidin-AlexaFluor647 to detect biotinylated proteins (middle row) and anti-V5 antibody (AlexaFluor 488 readout) to detect HRP construct expression (top row). Note that our staining conditions highlight the surface pool of biotinylated proteins, but the total pool of V5-tagged HRP constructs. The last two columns show negative controls with H\textsubscript{2}O\textsubscript{2} or BxxP omitted. DIC, differential interference contrast.

(B) Zoomed-in views of boxed regions in (A). Synaptic constructs show punctate BxxP labeling, whereas HRP-TM gives a diffuse staining pattern. Scale bars, 10 um.

(C) Streptavidin blot analysis of whole neuron lysates infected and labeled with BxxP as in Figure 2B. The three bands in the negative control lanes at 129, 81, and 79 kDa are endogenously biotinylated proteins (Chapman-Smith and Cronan, 1999).

(D) Relative expression levels of the HRP fusion constructs. Samples were prepared as in (C), then stained with anti-V5 antibody to visualize the HRP fusion constructs. Endogenous actin is stained in the same lysates for reference.
Figure S5. Further Analysis of the Proteomic Data, Related to Figure 3

(A) Scatterplots showing correlation of iTRAQ ratios across independent experiments.

(B) Venn diagrams showing overlap between datasets, after application of Filters 1 and 2, but before application of Filter 3.

(C) Table showing increase in specificity and decrease in coverage as data from independent experiments are intersected to produce excitatory synaptic cleft proteomes. As in (B), Filters 1 and 2 have been applied to these datasets, but not Filter 3. Here, specificity is defined as the fraction of proteins with synapse annotation in GOCC, Synaptome DB, the Biesemann et al., 2014 synaptosome MS study, the Bayès et al., 2012 PSD MS study, or the Boyken et al., 2013 active zone MS study. The specificity calculation here does not include additional proteins with literature connection to synapses discovered by manual searching, as we performed for our final proteomic list. Coverage is based on a true positive list of 62 well-established excitatory synaptic cleft proteins (Table S3, Tab 1).

(D) Same as scatterplot in Figure 4D, except for Experiment 1 and 3 datasets, instead of Experiment 2.
Figure S6. Fluorescence Imaging of Synapse Sub-Type Orphans and Analysis Of CD200 at Excitatory Synapses, Related to Figure 5

(A) The HRP tag is superior to Venus fluorescent protein for detection of surface proteins at low expression levels. The tagged BAI3 fusion constructs shown at top were introduced to cultured neurons in three expression level regimes: highest, via lipofectamine transfection; lower, via lentiviral transduction; and lowest, via lentiviral transduction but with 100-fold less virus. The last column is the same as the third column, but the intensity scale is narrowed to show the images at higher contrast. At DIV19, neurons were labeled live with BxxP, then fixed without permeabilization and stained with neutravidin-AlexaFluor647 to detect biotinylated proteins and anti-V5 antibody (AlexaFluor568 readout) to detect the V5 tag. Whereas HRP-BAI3 puncta are visible, Venus-BAI3 puncta cannot be detected in the lowest expression regime. Scale bar, 10 μm.

(B) Same as A, except the anti-V5 staining was performed after cell permeabilization, in order to detect total protein pools rather than cell surface pools only. Scale bar, 10 μm.

(C) Table summarizing data on synapse sub-type orphans, which are proteins known to be generally synaptic, but their synapse sub-type localization preferences are unknown or incomplete. E, excitatory. I, inhibitory.

(D) Imaging of synapse subtype orphans Flt2, Ephb6, and Dcc. Orphan genes were fused at their N-terminal ends to HRP, and expressed via lentiviral transduction in DIV19 dissociated rat cortical neurons. The HRP tag was visualized by live BxxP labeling, followed by neutravidin-AlexaFluor647 staining on fixed cells. Endogenous vGAT and vGlut1 were detected with respective antibodies followed by AlexaFluor488 and AlexaFluor568 readout.

(E) Imaging of Elfn1-GFP in DIV19 rat cortical neuron cultures (lentiviral transduction). vGAT and vGlut1 were detected as in (D). Within the same culture dish, Elfn1 appeared at excitatory synapses in some fields of view (left), and at inhibitory synapses in other fields of view (right). > 13 fields of view were analyzed per construct shown in (D) and (E); scale bars 10 μm; errors, ±1 s.d.

(F) Representative images of CD200 immunostaining in coronal sections of wild-type mouse brain. CD200 is localized throughout the neuropil and is most highly expressed during periods of synapse development and remodeling (left). Arrow denotes the dorsostral geniculate nucleus (dLGN). Scale bar, 1 mm. Magnified images of the dLGN demonstrate that CD200 levels peak at P10 in this region (right, red (AlexaFluor594)). Presynaptic marker vGlut2 labels retinogeniculate synapses and can be used to visualize the dLGN (right, green (AlexaFluor488)). Scale bar, 200 μm.

(G) Representative structured illumination microscopy (SIM) image of CD200 (red; AlexaFluor594) in the P10 dLGN showing colocalization with presynaptic vGlut2 (green; AlexaFluor488) and postsynaptic Homer (cyan; AlexaFluor647). Orthogonal views confirm CD200 colocalization with an excitatory synapse (right and bottom edges). Scale bar, 1 μm.

(H) Representative confocal images of pre- and postsynaptic markers of retinogeniculate synapses in the dLGN of P10 CD200 knock out (KO) mice and wild-type (WT) littermates. Scale bar, 5 μm. Note the reduced number of colocalized puncta (denoting synapses; AlexaFluor488 and AlexaFluor594) in the CD200 KO.

(I) Quantification of retinogeniculate synapse numbers in CD200 KO mice relative to synapse numbers in WT littermate controls. There are significantly fewer retinogeniculate synapses in the dLGN of P10 CD200 KO mice \(^{*} p < 0.05 (n = 4 \text{ WT and 5 CD200 KO}).\) Error bars, SEM.
Figure S7. Additional Data Related to Mdga1 and Mdga2, Related to Figure 6
(A) Localization of HRP-MDGA1 to both excitatory and inhibitory synapses. Samples were prepared and imaged as in Figure 6A. Colocalization was quantified from 8 fields of view; errors, ± 1 s.d; scale bar, 10 μm.

(B) Validation of NLGN2 synaptogenesis assay used in Figure 6B. Overexpression of either NLGN1 or NLGN2 leads to enhanced recruitment of both excitatory and inhibitory synaptic vesicles. Samples were prepared, imaged, and quantified as in Figure 6C. HRP-TM is used as a non-synaptogenic negative control. 8 fields of view were analyzed per condition. Errors, ± SEM; *** indicates p < 0.0001 (Student’s t test).

(C) Possible model for Mdga1 versus Mdga2 action via Nlg2 at synapses. Through unknown presynaptic binding partners, Nlg2 can recruit both inhibitory and excitatory presynaptic terminals (left panel). Mdga2 may bind to Nlg2 in cis to selectively downregulate its recruitment of excitatory vesicles but not inhibitory vesicles (middle). In contrast, Mdga1 may bind to Nlg2 in cis to downregulate its recruitment of both vesicle types (right panel). This activity may serve to both regulate inhibitory synapse size and prevent invasion of Nlg2 into excitatory synapses.

(D) Fluorescence images associated with knockdown and rescue experiment shown in Figure 6E. shRNAs and GFP marker were introduced by lentiviral transduction at DIV7, and rescue constructs (mApple-MDGA1 or mApple-MDGA2) were introduced by lentivirus at DIV11. At DIV15, neurons were fixed and stained with anti-vGlut1 and anti-vGAT antibodies. Scale bar, 10 μm.

(E) Imaging of Mdga1/Mdga2 chimeras. Related to Figures 6G-H. N-terminal HRP-tagged chimeras were imaged and quantified as in (A). Colocalization values are based on these images and an additional 5 fields of view not shown (with > 300 puncta) per construct. Errors, ± 1 s.d. Scale bars, 10 μm.