Functional Excitatory Synapses in HEK293 Cells Expressing Neuroligin and Glutamate Receptors

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Fu, Zhanyan, Philip Washbourne, Pavel Ortinski, and Stefano Vicini. Functional excitatory synapses in HEK293 cells expressing neuroligin and glutamate receptors. J Neurophysiol 90: 3950–3957, 2003. First published August 20, 2003; 10.1152/jn.00647.2003. The discovery that neuroligin is a key protein involved in synapse formation offers the unprecedented opportunity to induce functional synapses between neurons and heterologous cells. We took this opportunity recording for the first-time synaptic currents in human embryonic kidney 293 (HEK293) cells transfected with neuroligin and the N-methyl-b-aspartate or AMPA receptor subunits in a co-culture with rat cerebellar granule cells. These currents were similar to synaptic currents recorded in neurons, and their decay kinetics was determined by the postsynaptic subunit combination. Although neuroligin expression was sufficient to detect functional synapses, cotransfection of HEK293 cells with Postsynaptic density-95/synapse-associated protein-90 (PSD-95) significantly increased current frequency. Our results support the central role of neuroligin in the formation of CNS synapses, validate the proposal that PSD-95 allows synaptic maturation, and provide a unique experimental model to study how molecular components determine functional properties of excitatory synapses.

INTRODUCTION

Neuroligins 1–3 constitute a family of brain-specific cell-adhesion membrane proteins (Ichtchenko et al. 1990, 1996). It has been demonstrated that these molecules are localized postsynaptically at excitatory synapses and interact with β-neurexins, resulting in the formation of synaptic junctions (Dean et al. 2003; Nguyen and Sudhof 1997; Song et al. 1999). Using an in vitro system, Scheiffele et al. (2000) demonstrated that neuroligins can trigger morphological presynaptic differentiation in contacting axons. In this study, functional synapse formation was inferred by showing that neuroligin expressed in nonneuronal cells leads to clustering of synaptic vesicles within axons. These clusters displayed functional exocytosis as seen by increased staining with antibodies against the luminal domain of synaptotagmin after incubation in a depolarizing solution (Scheiffele et al. 2000). While this evidence strongly suggested functionality in newly formed synapses, it was nevertheless indirect. In our study, we tested for the presence of functional synapses directly by co-transfecting human embryonic kidney 293 (HEK293) cells with a GFP-tagged form of neuroligin and subunits of the N-methyl-b-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and verifying synaptic transmission electrophysiologically.

Under culture conditions favoring synaptic transmission, cerebellar granule cells (CGCs) can form a large excitatory network (Chen et al. 2000; Mellor et al. 1998; Virginio et al. 1995) that exhibits paroxysmal activity when Mg2+ is removed from the extracellular solution (ECS) (Chen et al. 2000; Losi et al. 2002). Application of TTX allows low-frequency NMDA and AMPA-mediated miniature excitatory postsynaptic currents (mEPSCs) to be recorded and studied (Losi et al. 2002). Over-expression of NR2A and NR2B NMDA receptor subunits in these cells determines the decay kinetics of NMDA EPSCs (Prybylowski et al. 2002). This matches, to some extent, data from rapid agonist applications to HEK293 cells transfected with NMDA receptor (NMDAR) subunits (Cull-Candy et al. 2001), indicating that the expression of the NR2A subunit is critical to produce fast decay kinetics of the response. Our experimental model of functional synapse formation between CGCs and HEK293 cells transfected with NMDA receptor subunits allows further investigation of this hypothesis by providing a unique opportunity to study how subunit composition of postsynaptic receptors sets the properties of excitatory synapses.

Postsynaptic density-95/synapse-associated protein-90 (PSD-95) is a member of the membrane-associated guanylate kinases (MAGUKs) superfamily (Garner et al. 2000; Kornau et al. 1997). In postsynaptic densities, the cytosolic C-terminal tails of NMDA receptor subunits associate with distinct PSD-95/discs large/zona occludens-1 (PDZ) domains (Garner et al. 2000; Kennedy 1998; Kornau et al. 1997). The first two PDZ domains of PSD-95 bind to the NMDA receptor subunits NR2A and NR2B, whereas the third PDZ domain interacts with the C terminus of neuroligins (Hunt et al. 1996; Irie et al. 1997). In addition, redistribution of PSD-95 from the cytosol to the plasma membrane can be induced by transfection of neuroligin 1 or NR2A subunits in HEK293 cells (Irie et al. 1997). Developmental expression of PSD-95 parallels that of NR2A subunit (Sans et al. 2000). At the same time, PSD-95 expression parallels that of neuroligin and of NR1 subunit during development in brain homogenates (Song et al. 1999). In hippocampal and cortical neurons, PSD-95 over-expression

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increases the AMPA mEPSCs and AMPA receptor expression at the synapse but does not affect NMDARs (Béique and Andrade 2003; El-Husseini et al. 2000; Schnell et al. 2002). However, functional consequences of a specific interaction between PSD-95 and NR2A subunits have been reported in visual cortical neurons as well as in CGCs (Losi et al. 2003; Towsend et al. 2003). In all these studies, PSD-95 effects were abolished by mutation of N-terminus cysteines into serines (PSD-95gfpC3,5S), preventing the palmityolation of the protein (Craven et al. 1999).

Taken together, these data suggest a specific interaction between neuroligin, PSD-95 and NMDAR. Indeed, neuroligins have been proposed to organize the postsynaptic assembly of protein complexes involved in signal transduction (Scheiffele et al. 2000; Song et al. 1999). We therefore investigated the effect of PSD-95 over-expression on NMDA-mEPSCs resulting from the functional synapse formation between CGCs and NMDAR expressing HEK293 cells cotransfected with neuroligin and PSD-95.

METHODS

DNA constructs

Neuroligin was amplified from cDNA obtained from occipital rat cortex RNA using the primers neuroligin1f (ctcaagcttatggcacttccca) and the above-mentioned primers. A SalI restriction site was incorporated into the sixth codon after the signal sequence splice site (gatgcatgtggc) and neuroligin1rev (ggtctcgagctataccctggttgttgaatgtz) and the above-mentioned primers. A SalI restriction site was thus introduced. GFP was amplified from the EGFP vector (Clontech, Palo Alto, CA) by using GFPf (ctctgagcatggtgagcaagggcgaggagc) and the above-mentioned primers. A SalI restriction site was incorporated into the sixth codon after the signal sequence splice site (gatgcatgtggc) and neuroligin1rev (ggtctcgagctataccctggttgttgaatgtz) and the above-mentioned primers.

CGC and HEK293 cell culture and transfection

Primary cultures of rat cerebellar granule neurons were prepared from postnatal day 7 (P7) Sprague-Dawley rats. Rat pups were killed by decapitation in agreement with the guidelines of the Georgetown University Animal Care and Use Committee. The cerebella were dissociated as described in Gallo et al. (1987). Cells were dispersed with trypsin (0.25 mg/ml, Sigma, St. Louis, MO) and plated at a density of 1.1 × 10^5 cells/ml on glass coverslips (Fisher Scientific, Pittsburgh, PA) coated with poly-l-lysine (10 μg/ml; Sigma) in 35-mm Nunc dishes. The cells were cultured in basal Eagle’s medium supplemented with 10% bovine calf serum, 2 mM glutamine, and 100 μg/ml gentamycin (all from Invitrogen, Carlsbad, CA) and maintained at 37°C in 5% CO2. The final concentration of KCl in the culture medium was adjusted to 25 mM (high K+). To achieve functional synapse formation, at DIV5 the medium was replaced with the low (5 mM) potassium medium (MEM supplemented with 5 mg/ml glucose, 0.1 mg/ml transferrin, 0.025 mg/ml insulin, 2 mM glutamine, 20 μg/ml gentamicin, Invitrogen and cytosine arabinofuranoside 10 μM, Sigma) as previously described (Chen et al. 2000; Pryzbylowski et al. 2002). Human embryonic kidney 293 cells (American Type Culture Collection, Rockville MD, ATCC No. CRL1573) were grown in Minimal Essential Medium (Gibco BRL, Gaithersburg, MD), supplemented with 10% fetal bovine serum, 100 units/ml penicillin (Gibco BRL), and 100 units/ml streptomycin (Gibco BRL) in a 5% CO2 incubator. Exponentially growing cells were dispersed with trypsin, seeded at 2 × 10^5 cells/35-mm dish in 1.5 ml of culture medium. HEK293 cells after transfection were dispersed with trypsin and plated on CGCs cultures at a density of 1 × 10^5 cells/12-mm coverslip. HEK293 cells were transfected as described in Vicini et al. (1998). Briefly, mixed plasmids (3 μg total) were added to the dish containing 1.5 ml MEM culture medium for 12–16 h at 37°C under 5% CO2. >80% of cells expressed all the plasmids transfected as assessed independently with Clontech pEGFP, pDsRED2, and pECFP plasmids (not shown).

Immunocytochemistry

All incubations for staining experiments were done at room temperature. Mixed CGCs-HEK293s cultures were fixed in 4% paraformaldehyde, 4% sucrose in PBS for 5 min, and then incubated in 0.3% Triton X-100 for 10 min. Cells were preincubated in 10% BSA (Sigma) for 1 h and then incubated with primary antibodies in phosphate-buffered saline (PBS) containing 3% BSA for 1 h. Rabbit anti-synapsin1 antibody (Chemicon, Temecula, CA) was used at 1:4,000. After washing with PBS for several times, cells were incubated for 1 h with indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) used at 1:2,000. Coverslips were mounted on slides using AntiFade component A (Molecular Probe, Eugene, OR) as mounting medium. The cultures were imaged on a Nikon EN600 microscope equipped with a 26×, 1.0 numerical aperture objective. Spectral characteristics of the excitation-emission filters used were 490/530 nm for GFP and 545/610 nm for Cy3. The camera used was a Hamamatsu Orca-100, 12-bit cooled CCD digital camera, 1,392 × 1,040 pixel array. Images were captured and pseudocolored for presentation with MetaMorph imaging software (Universal Imaging, Downingtown, PA) and Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). GFP puncta and antibody-positive clusters were defined as clusters of fluorescence that were at least twice the background fluorescence of the image. Co-localization of GFP and synapsin-positive puncta was defined as having overlapping pixels. All immunocytochemical analysis was done blindly.

Electrophysiology

The recording chamber was continuously perfused at 5 ml/min with ECS composed of (in mM) 145 NaCl, 5 KCl, 1 MgCl2, 1 CaCl2, 5 HEPES, 5 glucose, 25 sucrose, 0.25 mg/l phenol red, and d-serine (5 μM) (all from Sigma) at pH adjusted to 7.4 with NaOH. All experiments were performed at room temperature (24–26°C). The recording solution contained (in mM): 145 potassium gluconate, 10 HEPES, 5 glucose, 25 sucrose, 0.25 mg/l phenol red, and 0.2 g/l NaCl. For recordings of GABA-elicited currents, we used a recording solution with KCl replacing potassium gluconate. Electrodes were pulled in two stages on a vertical pipette puller from borosilicate glass capillaries (Worldwide II, Drummond, Broomall, PA). Pipette resistance ranged from 3 to 5 MΩ. NMDAR-mediated responses were pharma...
coliologically isolated by BMI (50 μM, Sigma) and NBQX (5 μM, Tocris). NMDA mEPSCs and AMPA spontaneous EPSCs (sEPSCs) were recorded at −60 mV in Mg2+-free solution in the presence or absence of 1 μM TTX, respectively. Solutions and drugs were superfused through parallel inputs to the perfusion chamber or locally perfused by means of a Y tube (Murase et al. 1989). DIV7–8 cells were used (1–2 days after HEK293 cells were plated on CGCs). Whole cell recordings were performed with a patch-clamp amplifier (Axopatch 200, Axon Instrument, Foster City, CA). CGCs and HEK293 cells were voltage clamped at −60 mV, and access resistance was monitored throughout the recordings. Capacitance was assessed from the transient current in response to a 10 mV hyperpolarizing pulse. Currents were filtered at 1 kHz with an 8-pole low-pass Bessel filter (Frequency Devices, Haverhill, MA), digitized at 5–10 kHz, and averaged currents were derived from 20 events per 5 min. As a control, we tested for the occurrence of paroxysmal currents as

\[ I(t) = I_\alpha \times \exp(-t/\tau_\alpha) + I_\beta \times \exp(-t/\tau_\beta), \]

where \( I_\alpha \) and \( I_\beta \) are the amplitudes of the fast and slow decay components, and \( \tau_\alpha \) and \( \tau_\beta \) are their respective decay time constants. To compare decay times between different subunit combinations we used a weighted mean decay time constant \( \tau_{\text{w}} = \frac{[I_\alpha/(I_\alpha + I_\beta)] * \tau_\alpha + [I_\beta/(I_\alpha + I_\beta)] * \tau_\beta}{I_\alpha + I_\beta} \). All data values are expressed as means ± SE unless otherwise indicated. P values represent the ANOVA for multiple comparisons, with \( P < 0.05 \) as significance threshold.

**RESULTS**

To investigate functional synapse formation between neurons and HEK293 cells, we co-cultured CGCs with HEK293 cells transfected with NR1-1a/NR2A and neuroligin-GFP cDNAs. CGCs cultured in low-potassium medium form functional inhibitory and excitatory synapses (Chen et al. 2000; Losi et al. 2002) beginning at DIV6. We therefore selected this time point to plate the transfected HEK293 cells and tested for presence of synaptic current in these cells the day after. As illustrated in Fig. 1 both CGCs and the transfected HEK293 cells displayed paroxysmal currents in a Mg2+-free ECS. These currents were completely abolished by the NMDA antagonist CPP (10 μM, Fig. 1A). This indicates that these currents were due to CGC network excitation caused by waves of poorly synchronized synaptic input triggered by Mg2+ removal. After perfusion with TTX (0.5 μM), the paroxysmal currents in both HEK293 cells and CGCs were replaced by infrequent NMDA mEPSCs as shown in Fig. 1 (quantified in Fig. 3D). However, in HEK293 cells, the frequency of these currents was very low, being <20 events per 5 min. As a control, we tested for the occurrence of paroxysmal currents as

**FIG. 1.** Neuroligin-GFP allows synapse formation in NR1-1a/NR2A transfected human embryonic kidney 293 HEK293 cells. A: representative recording from a HEK293 cell transfected with NR1-1a/NR2A and neuroligin-NLG-GFP. On removal of Mg2+ (top), N-methyl-D-aspartate (NMDA) receptor-mediated paroxysmal currents produced by a large number of synaptic inputs from many cerebellar granule cells (CGCs) were recorded. In the presence of TTX (0.5 μM, bottom), miniature excitatory postsynaptic currents (mEPSCs) mediated by NMDA receptors (NMDA-mEPSCs) were recorded. B: paroxysmal currents recorded from cocultured CGCs on perfusion with Mg2+-free extracellular solution (ECS; top), NMDA-mEPSCs were then recorded in Mg2+-free ECS containing TTX (0.5 μM), BMI (20 μM), and 2,3-di-hydro-6-nitro-7-sulfamoyl-benzo(F)quinoxaline (NBQX; 0.5 μM, bottom). Perfusion with CPP (10 μM) abolished all synaptic activity. C: a representative trace showing whole cell current produced when Mg2+ was removed locally in a control HEK293 cell transfected with NR1-1a/NR2A and GABAA receptor subunit combinations. D: example current elicited by GABA (100 μM) in a HEK293 cell transfected with NR1-1a/NR2A and GP with neuroligin-GFP. No spontaneous inhibitory synaptic currents were recorded in this cell. E: summary of percent HEK293 cells with detectable NMDA-mEPSCs transfected with NR1-1a/NR2A and neuroligin-GFP vs. NR1-1a/NR2A and GFP (n > 29 cells in 3 different experiments; * P < 0.05, ANOVA).
well as the occurrence of NMDA-mEPSCs in co-cultured HEK293 cells lacking neuroligin and expressing only NR1–1a/NR2A and GFP. As illustrated in Fig. 1C, removal of Mg^{2+} led to the increase of the background noise sometime with a wavering pattern. Paroxysmal currents were observed in only 1 of 29 neuroligin-lacking cells tested in three different sets of experiments (Fig. 1E). This number was dramatically increased in neuroligin-GFP transfected cells (15 of 32 cells in 4 experiments, Fig. 1E). As an additional control, we transfected HEK293 cells with the α1, β3, and γ2 subunits of GABA_A receptor together with neuroligin-GFP. After plating these cells on CGCs we were able to record spontaneous inhibitory synaptic currents from CGCs, but not from HEK293 cells (n = 15). In transfected HEK293 cells, GABA (100 μM) applications elicited currents (Fig. 1D) indicating a successful expression of GABA_A receptors.

To study the reported interactions between neuroligin and PSD-95, we expressed GFP-tagged versions of these proteins in CGC/HEK293 co-cultures. In Fig. 2, we illustrate examples of these co-cultures with differential interference Nomarski contrast (DIC) and with fluorescence microscopy. Neuriligin-GFP tends to outline the apparent edge of the cell, suggesting a plasma membrane localization, sometimes with elongated circular clusters (Fig. 2A). Consistent with previous reports, neuroligin caused morphological changes in HEK293 cells such as membrane ruffling and filopodial sprouting (Scheiffele et al. 2000). Co-transfection of neuroligin wild-type with PSD-95-gfp led to appearance of distinct puncta of fluorescence in HEK293 cells (Fig. 2B). In three sets of experiments, 89 ± 9% of HEK293 cells displayed the fluorescent puncta (n = 40 cells) in contrast to 8 ± 7% of HEK293 cells transfected with PSD-95-gfp alone (n = 34 cells). Transfection with wild-type PSD-95 and neuroligin-GFP did not lead to well defined cluster formation (n = 36 cells in 3 experiments). Co-transfection of neuroligin wild-type and a construct with mutation of N-terminal cysteines into serines in PSD-95 that prevents the palmitoylation of the protein (PSD-95-gfpC3,5S) (Craven et al. 1999) also did not result in the formation of fluorescent puncta (Fig. 2C, n = 28 cells in 4 experiments). These data taken together suggest that the fluorescent puncta observed are due to neuroligin-induced PSD-95-gfp clustering, although the two proteins do not display the same expression pattern.

Figure 3, A and B, illustrates examples of currents recorded from one of the HEK293 cells co-transfected with neuroligin-GFP/PSD-95-gfp and NR1–1a/NR2A cDNAs. The percent of cells displaying paroxysmal currents and NMDA-mEPSCs in Mg^{2+}-free ECS was higher compared with cells lacking PSD-95-gfp (Fig. 3C). The frequency of NMDA-mEPSCs increased by 220% with PSD-95-gfp expression (Fig. 3D). The amplitude of NMDA-mEPSCs, however, was not significantly different between cells transfected with neuroligin-GFP and neuroligin-GFP/PSD-95-gfp (Fig. 3D). Co-transfection of NR1–1a/NR2A, neuroligin-GFP and PSD-95-gfpC3,5S did not significantly increase the percent of cells displaying NMDA currents (Fig. 3C).

Currents recorded from one of the HEK293 cells co-transfected with neuroligin-GFP/PSD-95-gfp and NR1–1a/NR2B cDNAs are illustrated in Fig. 4, A and B. The percent of cells displaying paroxysmal currents and NMDA-mEPSCs on removal of Mg^{2+} was not significantly different from NR1–1a/NR2A transfected cells. In Fig. 4C, averages of 20 NMDA-mEPSCs are compared between cells transfected with neuroligin-GFP/PSD-95-gfp and NR1–1a/NR2A or NR1–1a/NR2B. The weighted decay time constant was remarkably slower for cells expressing the NR1–1a/NR2B subunit as reported with rapid agonist applications (Vicini et al. 1998). The averages of both the amplitude and the decay time constant were significantly different between the two groups (Fig. 4D).

NMDA-mEPSCs recorded in transfected HEK293 cells suggested synapse formation. However, given the high sensitivity of NMDARs to glutamate it is possible that presynaptic terminal and postsynaptic receptors in such synapses are not precisely aligned and synaptic currents may be generated by spillover from synapses on neighboring CGCs. AMPA receptors are less sensitive to glutamate and thus require a close apposition of the release site (Gasparini et al. 2000). To assess the alignment of pre- and postsynaptic sites, we transfected HEK293 cells with the GluRDflip subunit of AMPA receptors together with neuroligin-GFP and PSD-95-gfp (Fig. 5). Recombinant AMPA receptors containing this subunit have very fast desensitization, and neurons expressing GluRDflip have been reported to produce very fast decaying synaptic events (Dingledine et al. 1999). In 25 of 39 cells in four separate experiments, we detected the occurrence of sEPSCs mediated by recombi-
nant AMPA receptor (Fig. 5), suggesting a good alignment between pre- and postsynaptic sites. In 3 of 12 cells cotransfected with GluRDLflip and PSD-95gfplp and in 1 of 11 cells transfected with GFP we could only record AMPA-sEPSCs in the presence of cyclothiazide (50 μM), a compound that enhances glutamate release and removes AMPA receptor desensitization (Diamond and Jahr 1995; Ishikawa and Takahashi 2001; Yamada and Tang 1993). This indicates that poor alignment of synapses or glutamate spillover from neighbor synapses does occur in a minority of cells in the absence of neuroligin. We also investigated the amplitude distribution of mEPSCs in one transfected HEK293 cell. As seen in Fig. 5D, this distribution was skewed to the left, similarly to what is observed in neurons (Frerking et al. 1997). This suggests that postsynaptic receptor clustering is regulated similarly in neurons and transfected HEK293 cells with a larger abundance of clusters with small number of receptors giving rise to smaller synaptic currents.

To further investigate the alignment between pre- and postsynaptic sites in CGC-HEK293 cell cultures, we performed immunocytochemical staining with antibodies against the presynaptic marker synapsin 1 (Fig. 6). Synapsin1 has been shown to accumulate at newly formed contact sites in similar systems (Schieffele et al. 2000). The percentage of PSD-95gfplp puncta colocalized with synapsin significantly increased from 17 ± 3% (n = 10 cells) to 56 ± 6% (n = 13 cells, P < 0.05 ANOVA) when cells were cotransfected with neuroligin supporting the alignment between pre- and postsynaptic sites.

**Discussion**

We demonstrated with voltage-clamp recordings the formation of functional excitatory synapses between CGCs and HEK293 cells expressing glutamate receptors. Electrophysiological studies of recombinant ligand-gated ion channels mediating synaptic transmission expressed in heterologous systems have greatly contributed in the last 20 years to the understanding of the molecular determinants of postsynaptic receptors. Our results on the reconstitution of functional synapses on HEK293 cells are a step forward that will allow insights into the molecular mechanisms underlying synaptic function in the CNS.

Expression of neuroligin greatly facilitated the formation of functional excitatory synapses between neurons and heterologous cells as previously reported (Dean et al. 2003; Schieffele et al. 2000). Spontaneous paroxysmal currents and NMDA-mEPSCs recorded from neuroligin-transfected HEK293 cells were likely due to the vesicular release from contacting axons of the CGCs. Arguing against neuroligin-mediated synaptic connection between CGCs and HEK293 cells, however, one can hypothesize random contact of the presynaptic axons/growth cones with HEK293 cells, which could have allowed us to record synaptic events. In addition, large axonal plexuses reported in CGC cultures (Leao and Randall 2000) may cause spillover onto the neighboring HEK293 cells likewise eliciting synaptic currents. Waves of NMDAR activation were indeed observed in control cells transfected only with GFP and the NMDAR subunits indicating the occurrence of spillover. However, in only a few of these cells did we observe better defined synaptic events, as opposed to the presence of such events in half the cells expressing neuroligin. These results imply that neuroligin is at least greatly facilitating the molecular recognition between growing excitatory axons and their postsynaptic target. Independent support of our results comes from studies showing that neuroligin-1 is concentrated at the postsynaptic...
side of glutamatergic but not GABAergic synapses (Song et al. 1999). Indeed, after co-transfection of GABA A receptor subunits with neuroligin, we failed to detect formation of GABAergic synapses in HEK293/CGC co-culture. We speculate that our model will be useful for testing putative proteins involved in the formation of inhibitory synapses and determining their roles.

It has been suggested that the neurexin–neuroligin interaction may also mediate postsynaptic differentiation (Rao et al. 2000). Indeed, neuroligin C-terminus binds two major scaffolding molecules, PSD-95 and S-SCAM, that link neuroligins to NMDA receptors and downstream signal-transducing proteins (Hirao et al. 1998, 2000; Irie et al. 1997). Neuroligin also induces morphological changes in HEK293 cells (Scheiffele et al. 2000) suggestive of an action on the actin cytoskeleton possibly mediated by Rho GTPase (Cantallops and Cline 2000; Van Aelst and D’Souza-Schorey 1997). Similarly, morphological differentiation of presynaptic components and neurexin clustering by neuroligin has been recently demonstrated in both CGCs and hippocampal neurons (Dean et al. 2003; Missler et al. 2003). In addition, Irie et al. (1997) demonstrated that membrane redistribution of PSD-95 can be induced by neuroligin 1 or the NR2A subunit in transfected cells. Our results indicate that clusters of PSD-95gfp can be observed in the transfected CGCs/HEK293 co-cultures. These clusters are likely morphological correlates of functional synapse formation as PSD-95 (but not PSD-95C3,5S) increases the number of cells displaying synaptic events and increases the frequency of NMDA-mEPSCs. This supports the reported evidence that the major role of PSD-95 is to allow the maturation of the synapse (El Husseini et al. 2000). We speculate that the interaction of PSD-95 with neuroligin is sufficient and perhaps necessary to allow for the formation of synapses that are electrophysiologically indistinguishable from mature neuronal synapses.

Immunocytochemical staining with synapsin specific antibodies demonstrated that neuroligin facilitates the juxtaposition of the postsynaptic PSD-95 clusters on HEK293 cells and the presynaptic axon terminals of the contacting CGCs. Similarly to currents recorded in neurons, the occurrence of fast synaptic currents in HEK293 cells transfected with an AMPA...
receptor subunit supports the morphological evidence of pre- and postsynaptic juxtaposition. Taken together these data support the suggestion of the neurexin–neuroligin link as a signaling device triggered by initial contact that leads to functional synapses (Rao et al. 2000).

Because the contribution of glutamate receptor subtypes to shape the postsynaptic current characteristics is a fundamental question (Cull-Candy et al. 2001; Dingledine et al. 1999), verifying the role of subunit heterogeneity by using defined subunit compositions is of the utmost importance. Ultra-rapid application to patches excised from HEK293 cells expressing subunit compositions is of the utmost importance. Ultra-rapid verifying the role of subunit heterogeneity by using de

FIG. 6. Neuroligin increases co-localization of PSD-95gfp with synapsin. Immunostaining with antisynapsin 1 antibodies (Cy3 secondary Ab) of CGCs and HEK293 cells co-cultures. HEK293 cells were transfected with PSD95-gfp with (B) or without (A) neuroligin. Lower panels show overlays. Calibration bar: 4 μm.

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DISCLOSURES

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