Functional Excitatory Synapses in HEK293 Cells
Expressing Neuroligin and Glutamate Receptors

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ABSTRACT

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 HEK293 cells transfected with neuroligin and the NMDA 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. While neuroligin expression was sufficient to detect functional synapses, cotransfection of HEK293 cells with 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.

Key Words: cerebellar granule cells, PSD-95, synapse formation, kinetic
INTRODUCTION

Neuroligins 1, 2, and 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 (Nguyen and Sudhof 1997; Song et al. 1999; Dean et al. 2003). 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 HEK293 cells with a GFP-tagged form of neuroligin and subunits of the N-methyl-D-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 (Virginio et al. 1995; Mellor et al. 1998; Chen et al. 2000) that exhibits paroxysmal activity when Mg\(^{2+}\) 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 (Kornau et al. 1997; Garner et al. 2000). In postsynaptic densities the cytosolic C-terminal tails of NMDA receptor subunits associate with distinct PSD-95/disc large/zona occludens-1 (PDZ) domains (Kornau et al. 1997; Kennedy 1998; Garner et al. 2000). 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 increases the AMPA mEPSCs and AMPA
receptor expression at the synapse, but does not affect NMDA receptors (El-Husseini et al. 2000; Schnell et al. 2002; Beique and Andrade 2003). 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 (Towsend et al. 2003; Losi 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 palmitoylation 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.
MATERIALS AND METHODS

DNA constructs

Neuroligin was amplified from cDNA obtained from occipital rat cortex RNA using the primers neuroligin1f (ctcaagcttatggcacttcccagatgtggc) and neuroligin1rev (ggtctcgagctataccctgtatggatgaatggggg). Subsequently, a single base change (silent mutation) was incorporated into the 6th codon after the signal sequence splice site (gta>gtc) by megaprimer PCR using Nlg1-MP (gtaaccaatgggctgacatcatccaac) and the above mentioned primers. A Sal I restriction site was thus introduced. GFP was amplified from the EGFP vector (Clontech, Palo Alto, CA) by using GFPf (ctcgtcgacatggtgagcaagggcgaggc) and GFPr (ggtgtcgacctgtacagctcgtccatgccg) to incorporate Sal I restriction sites at the 5’ and 3’ termini, and GFP was then inserted into the mutated neuroligin. PSD-95gfp and PSD-95gfpC3,5S were generous gifts of Dr. David Bredt (University of California, San Francisco) and are described in Craven et al. (1999). The NR1-1a, NR2A and NR2B constructs were described in Vicini et al. (1998). The GluRDflip construct subcloned in the PRK7 expression vector was a gift from Dr. Peter H. Seeburg (University of Heidelberg Germany). In some experiments we used the rat NR2B-Flag and the human NR2A-flag (NR2B or NR2A subunit tagged at the N-terminus with Flag epitope), the respective gifts of Dr. Anne Stephenson (School of Pharmacy University College, London) and Dr. Paul Whiting (Merck Sharp and Dohme, United Kingdom), which have been previously described (Hawkins et al. 1999; Prybylowski et al. 2002; McIlhinney et al. 1998). The human NMDAR subunits are highly conserved with over 95% homology to the NMDAR subunits in the rat. No significant differences were seen when flag-tagged subunits were used. Rat α1, β3 and γ2
GABA<sub>A</sub> receptor subunit cDNAs singly subcloned into the expression vector pCDM8 (Invitrogen) were provided by Dr. Peter H. Seeburg.

**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 sacrificed 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 1.1x10<sup>6</sup> 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 Corporation Carlsbad, CA), and maintained at 37°C in 5% CO<sub>2</sub>. The final concentration of KCl in the culture medium was adjusted to 25 mM (high K<sup>+</sup>). 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; Prybylowski 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% CO<sub>2</sub> incubator. Exponentially growing cells were dispersed with trypsin, seeded at
2x10^5 cells/35-mm dish in 1.5 ml of culture medium and plated on 12 mm glass cover slips. HEK293 cells after transfection were dispersed with trypsin and plated on CGCs cultures at a density of 1x10^4 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 hours at 37 °C under 5% CO2. Greater than 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 pre-incubated in 10% BSA (Sigma) for 1 hr and then incubated with primary antibodies in phosphate-buffered saline (PBS) containing 3% BSA for 1 hr. Rabbit anti-synapsin1 antibody (Chemicon, Temecula, CA) was used at 1:4000. After washing with PBS for several times, cells were incubated for 1 hr with indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch laboratories, West Grove, PA) used at 1:2000. 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 60x, 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, 1392 x 1040 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 blinded.

**Electrophysiology**

The recording chamber was continuously perfused at 5 ml/min with ECS composed of (in mM): NaCl (145), KCl (5), MgCl$_2$ (1), CaCl$_2$ (1), HEPES (5), glucose (5), sucrose (25), phenol red (0.25 mg/l) 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): potassium gluconate (145), HEPES (10), ATP.Mg (5), GTP.Na (0.2), and BAPTA (10), adjusted to pH 7.2 with KOH. For recordings of GABA-elicted 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 (Wiretrol II, Drummond, Broomall, PA). Pipette resistance ranged from 3 to 5 MΩ. NMDA receptor mediated responses were pharmacologically isolated by bicuculline methiodide (BMI, 50 µM, Sigma) and 2,3-Dihydro-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX, 5 µM, Tocris). NMDA mEPSCs and AMPA sEPSCs were recorded at –60 mV in Mg$^{2+}$-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 using an IBM-compatible microcomputer equipped with Digidata 1200 data acquisition board and pCLAMP9 software (both from Axon Instruments). Off-line data analysis, curve fitting, and figure preparation were performed with Clampfit 9 (Axon Instruments) software. Miniature synaptic currents were identified using a semi-automated mini detection software (Minianalysis, Synaptosoft, Decatur GA) with threshold criteria of 7.5 pA, three times greater than the RMS noise level and approximately equivalent to two overlapping channel openings. NMDA mEPSC averages were based on at least 20 events in each cell studied. Fitting of the decay phase of currents recorded from HEK293 cells was performed using a simplex algorithm for least squares exponential fitting routines. The decay times of averaged currents were derived from fitting to double exponential equations of the form I(t) = I_f X exp(-t/τ_f) + I_s X exp(-t/τ_s), where I_f and I_s are the amplitudes of the fast and slow decay components, and τ_f and τ_s are their respective decay time constants. To compare decay times between different subunit combinations we used a weighted mean decay time constant τ_w = [I_f/(I_f+I_s)] * τ_f + [I_s/(I_f+I_s)] * τ_s. All data values are expressed as mean ± SEM unless otherwise indicated. p values represent the analysis of variance (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 Mg\(^{2+}\)-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 Mg\(^{2+}\) 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 less than 20 events per 5 minutes. As a control, we tested for the occurrence of paroxysmal currents as 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 waving pattern. Paroxysmal currents were observed in only 1 out of 29 neuroligin-lacking cells tested in 3 different sets of experiments (Fig. 1E). This number was dramatically increased in neuroligin-GFP transfected cells (15 out 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\textsubscript{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. Neuroligin-GFP tends to outline the apparent edge of the cell suggesting a plasma membrane localization, sometimes with elongated thickening of fluorescence intensity but not well defined small 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-95gfp, 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-95gfp 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-terminus cysteines into serines in PSD-95 that prevents the palmitoylation of the protein (PSD-95gfpC3,5S, Craven et al. 1999) also did not result in the formation of fluorescence 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-95gfp clustering, although the two proteins do not display the same expression pattern.
Figures 3A and 3B illustrate examples of currents recorded from one of the HEK293 cells co-transfected with neuroligin-GFP/PSD-95gfp and NR1-1a/NR2A cDNAs. The percent of cells displaying paroxysmal currents and NMDA-mEPSCs in Mg\(^{2+}\)-free ECS was higher compared to cells lacking PSD-95gfp (Fig. 3C). The frequency of NMDA-mEPSCs increased by 220\% with PSD-95gfp expression (Fig. 3D). The amplitude of NMDA-mEPSCs, however, was not significantly different between cells transfected with neuroligin-GFP and neuroligin-GFP/PSD-95gfp (Fig. 3D). Co-transfection of NR1-1a/NR2A, neuroligin-GFP and PSD-95gfpC3,5S, 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-95gfp and NR1-1a/NR2B cDNAs are illustrated in figures 4A and 4B. The percent of cells displaying paroxysmal currents and NMDA-mEPSCs upon 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-95gfp 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 post-synaptic sites we transfected HEK293 cells with the GluRDflip subunit of AMPA receptors together with neuroligin-GFP and PSD-95gfp (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 out of 39 cells in 4 separate experiments we detected the occurrence of sEPSCs mediated by recombinant AMPA receptor (Fig. 5) suggesting a good alignment between pre- and post-synaptic sites. In 3 out of 12 cells co-transfected with GluRDflip and PSD-95gfp and in 1 out 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 (Yamada and Tang 1993; Diamond and Jahr 1995; Ishikawa and Takahashi 2001). 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 post-synaptic 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 experiments (Scheiffele et al. 2000). The percentage of PSD-95gfp 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 twenty 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 central nervous system.

Expression of neuroligin greatly facilitated the formation of functional excitatory synapses between neurons and heterologous cells as previously reported (Schieffele et al. 2000; Dean et al. 2003). 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 pre-synaptic 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 NMDA receptor activation were indeed observed in control cells transfected only with GFP and the NMDA receptor 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/CGL 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 (Van Aelst and D'Souza-Schorey 1997; Cantallops and Cline 2000). 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).

Since 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 NMDA receptors (Lester and Jahr 1992; Vicini et al. 1998) has demonstrated unique deactivation kinetics of distinct subtypes of NMDA receptors. The data presented here on the distinct decay kinetics between cells transfected with NR1-1a/NR2A and NR1-1a/NR2B are in line with these findings. A similar approach will allow the study of synaptic activation of several distinct AMPA and NMDA receptor subtypes. The results we obtained with sEPSCs in GluRDflip transfected cells indicate
that very fast decaying synaptic events can be recorded when this subunit of AMPA receptor is expressed at postsynaptic sites, as seen in single cell PCR studies of neurons expressing this subunit (Dingledine et al. 1999 for review).

Our work extends previous studies that had shown only morphological evidence for synapses between neuronal and non-neuronal cells expressing neuroligin and provides a unique model for studying the molecular elements required for synapse formation and function.
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FIGURE LEGENDS

Figure 1. Neuroligin-GFP allows synapse formation in NR1-1a/NR2A transfected HEK293 cells

A. Representative recording from a HEK293 cell transfected with NR1-1a /NR2A and neuroligin(NLG)-GFP. Upon removal of Mg$^{2+}$ (upper panel). NMDA receptor-mediated paroxysmal currents produced by a large number of synaptic inputs from many CGCs were recorded. In the presence of TTX (0.5 µM, lower panels) miniature EPSCs mediated by NMDA receptors (NMDA-mEPSCs) were recorded.

B. Paroxysmal currents recorded from cocultured CGCs upon perfusion with Mg$^{2+}$ free ECS (upper panel). NMDA-mEPSCs were then recorded in Mg$^{2+}$-free ECS containing TTX (0.5 µM, BMI (20 µM) and NBQX (0.5 µM, lower panels). Perfusion with CPP (10 µM) abolished all synaptic activity.

C. A representative trace showing whole-cell current produced when Mg$^{2+}$ was removed locally in a control HEK293 cell transfected with NR1-1a /NR2A and GFP.

D. Example current elicited by GABA (100 µM) in a HEK293 cell transfected with the α1, β3, and γ2 subunits of GABA$_{A}$ receptor together 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).

Figure 2. Neuroligin induces clusters of PSD-95 in transfected HEK293 cells co-
cultured with CGCs.

DIC microphotographs illustrating examples of CGCs/HEK293 cells co-cultures (left
column). In the right column, GFP fluorescence from cells shown on the left transfected
with neuroligin-GFP (NLG-gfp, A), with neuroligin wild type and PSD95-gfp (PSD-
95gfp, B) and neuroligin wild type and PSD-95gfpC3,5S (PSD-95gfpC3,5S, C).

Cotransfection of neuroligin-GFP and PSD95-wild type was not different from
transfection of neuroligin-GFP alone. Boxed regions in right column panels are shown
magnified in insets. Calibration bars: 20 µm and 6 µm for the insets.

Figure 3. PSD-95gfp increases NMDA-mEPSCs frequency in HEK293 cells
cotransfected with NR1-1a/NR2A and neuroligin-GFP

A. Representative currents recorded from HEK293 cells transfected with NR1-1a /NR2A
and neuroligin (NLG)-GFP and PSD-95gfp with ECS in the absence of Mg^{2+}.

B. Frequent NMDA-mEPSCs were recorded in Mg^{2+}-free ECS containing TTX.

C. Summary of percent HEK293 cells with detectable NMDA-mEPSCs for the different
transfections (n>19 cells in 3 different experiments), * p<0.05, ANOVA with respect to
PSD-95gfp transfection, ** p<0.05, ANOVA with respect to neuroligin-GFP (NLG)
transfection.
D. Summary of weighted decay time constant (Tw), peak amplitude (Peak) and frequency of occurrence in 5 min (Freq/5 min) of NMDA-mEPSCs recorded from HEK293 cells transfected with neuroligin-GFP (NLG) as compared to HEK293 cells transfected with neuroligin-GFP and PSD-95gfp (NLG+PSD95). The frequency of NMDA-mEPSCs increased significantly in cells cotransfected with PSD-95gfp compared to those only transfected with NLG-gfp (means ±S.E.M., n>44 cells in 5 different experiments; * p<0.05, ANOVA).

Figure 4. NMDA-mEPSCs decay is slower in HEK293 cells expressing NR2B than NR2A subunit.

A. Currents recorded in the absence of Mg$^{2+}$ from HEK293 cells transfected with NR1-1a/NR2B, PSD-95gfp and NLG-gfp.

B. NMDA-mEPSCs were recorded in the same cell after perfusion with ECS containing TTX in the absence of Mg$^{2+}$.

C. Comparison of NMDA-mEPSCs averages with superimposed double exponential fitting and indication of resulting weighted time constant (Tw) of decay for NR1-1a/NR2A (left panel) and NR1-1a/NR2B (right panel) transfected HEK293 cells.

D. Summary of Tw (ms) from HEK293 cells transfected with NR1-1a/NR2A (n=26) vs NR1-1a/NR2B (n=15; * p<0.05, ANOVA). All cells were co-transfected with PSD-95gfp and NLGgfp.

Figure 5. Synapse formation in HEK293 cells expressing GluRDflip with NLG-gfp and PSD-95gfp
A. Currents recorded in the absence of Mg\textsuperscript{2+} from HEK293 cells transfected with GluRDflip, PSD-95gfp and NLG-gfp.

B. High frequency sEPSCs were recorded in the same cell after some time with perfusion with ECS in the absence of Mg\textsuperscript{2+}.

C. sEPSCs average (15 events) with superimposed single exponential fitting and indication of resulting decay time constant (T).

D. Histogram distribution of amplitude of sEPSCs recorded from the cell illustrated in A-C (n=356 events).

E. Summary of decay time constant (T), peak amplitude (Peak), and frequency of occurrence (Freq) of sEPSCs recorded from HEK293 cells transfected with GluRDflip, NLG-gfp and PSD-95gfp (n>25 cells in 4 different experiments).

Figure 6. Neuroligin increases co-localization of PSD-95gfp with synapsin.

Immunostaining with antisynapsin 1 antibodies (Cy3 secondary Ab) of CGCs and HEK293 cells cocultures. HEK293 cells were transfected with PSD95-gfp with (B) or without (A) neuroligin. Lower panels show overlays. Calibration bar: 4 µm.
**Fig. 1 Fu et al.**

**A**

NLG-gfp HEK

Mg$^{2+}$ free

- +TTX
- +CPP

**B**

CGC

Mg$^{2+}$ free

- +TTX, BMI & NBQX
- +CPP

**C**

GFP HEK

Mg$^{2+}$ free

- 70 pA

**D**

NLG-gfp & $\alpha_1\beta_3\gamma_2$ HEK

GABA

- 2 nA

**E**

% cells

*NLG-GFP*

*GFP*
Fig. 3 Fu et al.

A

PSD-95gfp & NLG-gfp & NR1-1a/NR2A

Mg²⁺ free

B

+TTX

C

% cells

D

Tw (ms) Peak (pA) Freq/5 min

PSD-95gfp & NLG-gfp & NR1-1a/NR2A

Mg²⁺ free

* *
Fig. 4 Fu et al.

A. NR1-1a/NR2B

B. +TTX

C. NR1-1a/NR2A

D. NR2B

Peak (pA) Tw (ms)