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Identification of gene transcripts expressed by postsynaptic neurons during synapse formation encoding cell surface proteins with presumptive synaptogenic activity

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Abstract

Synapse formation is a well-programmed developmental process involving a variety of cell-cell interactions carried out by distinct groups of molecules. Various molecules that contribute to the assembly of synaptic contacts have been characterized; however, the repertoire of identified proteins expressed by postsynaptic neurons capable of inducing presynaptic differentiation is quite limited. To identify gene transcripts encoding cell surface proteins expressed by postsynaptic cells with molecular features suggestive of synaptogenic activity, this study carried out a genome-wide expression analysis in the chick ciliary ganglion during the different phases of synapse formation. It was found that from the 21,493 gene-probes detected throughout development, 302 protein-coding transcripts were up-regulated during the initiation of synapse formation. Analysis of this pool of transcripts showed that fifty one of them encoded cell surface proteins (27 membrane-bound and 24 secreted) with protein-protein interacting domains. This include, twelve cell adhesion molecules, six ligand-receptors, six proteins with ligand-like domains, three membrane bound enzymes, eight components of the extracellular matrix, three neuropeptides, three cytokines and growth factors, five extracellular modulators of cell signaling, and five unrelated secreted proteins. Furthermore, the role of synaptic transmission during the initiation of synapse formation was evaluated by assessing the effect of synaptic activity blockade with d-tubocurarine on the expression levels of the pool of fifty one transcripts encoding cell surface proteins. Treatment with d-tubocurarine reduced the expression levels of 22% of the selected genes, while the expression levels of 78% of the genes was not affected or was enhanced.

Keywords

Synaptogenesis; Gene expression analysis; Synaptic activity blockade; Ciliary ganglion; Cell adhesion molecules; Extracellular matrix

Introduction

The synapse is a highly specialized asymmetric structure comprised of a presynaptic terminal containing the molecular machinery for neurotransmitter release, and a postsynaptic compartment possessing the molecules required for signal transduction (Scheiffele, 2003). The formation of a synaptic contact involves a well-orchestrated sequence of events carried out by cell-cell interactions that lead to the structural and functional coupling between synaptic compartments (Akins and Biederer, 2006; Garner et al., 2006). This sequence of

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events can be divided into two main phases. The first phase or initiation of synapse formation involves interactions between presumptive synaptic compartments that induce the targeting of components of the synaptic machinery and the stabilization of the contact between cell membranes. These events require molecular recognition to determine the specific location for the formation of the synapse, and intercellular adhesion to promote cell-cell contacts (McAllister, 2007). The second phase of synaptic differentiation and maturation, involves the morphological specialization of synaptic terminals to best suit the physiological properties of particular synaptic types, and is characterized by the accumulation of synaptic components and the development of regulatory mechanisms necessary for efficient synaptic transmission (McAllister, 2007; Waites et al., 2005).

The different phases of synapse development are control by the successive activation of genetic and cellular programs resulting in the coordinated expression of gene products at both sides of the synaptic cleft (Diaz et al., 2002). On the one hand, presynaptic cells express surface receptors or release factors including agrin and neuregulin that recognize and modify the postsynaptic targets and promote postsynaptic differentiation (Corfas et al., 1993; Gautam et al., 1996). On the other hand, postsynaptic cells express cell surface and secreted ligands that bind to receptors on the presumptive synaptic membrane which promote the stabilization of the cellular contact and induce the differentiation of the presynaptic terminal. The best-known examples of this type of molecules are the transmembrane receptors neuroligins and synCAMs (Biederer et al., 2002; Scheiffele et al., 2000). In addition, secreted proteins including the fibroblast growth factor (FGF)-22, are capable of inducing presynaptic differentiation in a variety of neuronal types (Umemori et al., 2004). Finally, cell adhesion molecules (CAMs) are expressed by both pre and postsynaptic cells and are necessary for the stabilization of the synaptic contact (Schuster et al., 1996), and contribute to the structural and functional coupling between synaptic compartments (Brusés, 2006; Dalva et al., 2007).

Despite the importance of understanding the molecular mechanisms that control the initiation and differentiation of synapse development, the identification and characterization of molecules capable of inducing synaptogenesis has been limited. This is in part due to the lack of comprehensive information about the molecules expressed by postsynaptic neurons with potential synaptogenic activity. As the association between an increase in gene expression levels and a cellular function serves as an indication that the gene product is involved in that particular cellular process (Diaz et al., 2002; Eisen et al., 1998), this study carried out a genome-wide analysis of gene expression profiles during synapse formation in the chick ciliary ganglion with the goal of identifying gene transcripts encoding proteins expressed by postsynaptic neurons which may be capable of initiating the formation of synaptic contacts. Furthermore, as synaptic activity influences the development of neuronal connectivity (Verhage et al., 2000; Zhang and Poo, 2001), the effect of synaptic activity blockade on gene expression profiles during the induction of synapse formation was analyzed.

Materials and Methods

Animals

White Leghorn fertile chicken eggs were obtained from SPAFAS (Roanoke, IL) and incubated in a forced-draft incubator at 39°C under a humidified atmosphere until the desired developmental stage (St) was reached (Hamburger and Hamilton, 1951).

Microarray Analysis

Ciliary ganglia from chick embryos of the desired developmental age were dissected in cold Tyrode's solution (NaCl, 134 mM; KCl, 3 mM; NaHCO₃, 20 mM; CaCl₂, 3 mM; MgCl₂, 1 mM; glucose, 12 mM), immersed in liquid nitrogen, and stored at -80°C. Total RNA was isolated and cleaned using RNA isolation kits (Qiagen, Valencia, CA) from a pool of 50–100 ciliary ganglia at each developmental stage. Purity and integrity of the RNA from each sample was determined by spectrophotometry and capillary gel electrophoresis. The amount of RNA was normalized between samples and the same amount of RNA from each sample (>50ng) was reverse transcribed, labeled, and used to hybridize a GeneChip® Chicken Genome Array (Affymetrix, Santa Clara, CA) for 16 hrs at 45°C/60 rpm in a GeneChip hybridization oven 640. Hybridized GeneChips underwent low and high stringency washing and R-Phycoerythrin-Streptavidin staining procedures using the GeneChip fluidics station 450. The GeneChips were scanned in an Affymetrix Genechip scanner 3000 7G with autoloader. Microarray analysis of each stage was done in triplicates using individual tissue collection and RNA isolation for each sample. To examine whether changes in gene expression levels detected by the microarrays were consistent with changes in protein expression levels, the amount of four proteins in ciliary ganglion extracts from different developmental stages were determined by Western blot analysis and optical densitometry (data not shown). The correlation coefficient between gene expression and protein expression levels was between 0.98 and 1 (PTP-RB, 0.98; CDH2, 1.0; CTNNB1, 1.0; ITGB4, 0.98) indicating that changes in gene expression levels reflect changes in protein expression levels.

Synaptic activity was blocked by treatment with (+)-Tubocurarine chloride hydrate (Sigma T2379)(Sigma, St. Louis, MO). The shell of the eggs was windowed after 1 day of incubation and 1 mg of d-tubocurarine diluted in Tyrode's solution (10mg/ml) was applied daily from E5 to E7. At E8, The effectiveness of cholinergic blockade was determined by assessing the frequency of spontaneous hindlimb kicks. The ciliary ganglia from embryos having ≤3 kicks/min were collected and used for isolating RNA and gene expression analysis.

Analysis of Microarray Data

GeneChip microarrays analysis was carried out in triplicates of each developmental time points (E5, E8, E10 and E17), and from one time point (E8) treated with d-tubocurarine. The initial data captured by Affymetrix software resulted in a single raw value for each probe set based on the mean of the differences between the intensity of hybridization for a perfect match and the mismatch features for a specific transcript. Expression analysis data was exported via Data Transfer Tool into a data library by the Affymetrix GeneChip Operating Software. The Data Mining Tool was used for data quality control and to create subsets of expressed probes. The complete set of 38,535 probes for the chicken genome was divided into 'present', 'marginal' and 'absent' subsets based on the detection p-value. When the detection p-value (Student's T-test) was less than 5% (<0.05), the probe was considered as present (>95% significance). Probes with detection p-values between 5% and 6.5% were considered marginal, and probes with detection p-values >6.5% were designated as absent. Present and absent probes across all chips showed very similar distribution (~50%) while the marginal probes represent only small fraction of probes (<1.7%).

To determine which genes were up-regulated, only 'present' probes in the treatment versus all probes in the control were used, while for down-regulated genes probes 'present' in control were used versus all probes in the treatment set. GeneChip data from Affymetrix were then transferred into text file inputs for the GeneSpring software (Agilent Technologies, Santa Clara, CA). Two-steps normalization procedures were used: 1)

normalization per chip, which normalized each chip to the 50th percentile of the measurements taken from that particular chip, and 2) normalization per gene, which normalizes all samples per gene with respect to control by dividing each gene in the selected sample(s) by the median of that gene's measurements in the control sample(s). Appropriate time points (i.e. groups 1–4) or pharmacological treatment for each chip was then selected as experimental parameters. GeneSpring software was used for graphical interpretation of data and full gene annotation and gene ontology for biological processes, cellular components, and molecular functions.

Results

The chick ciliary ganglion is an anatomically distinct structure containing the presynaptic terminals and the postsynaptic cell bodies of a peripheral cholinergic synapse. The ciliary ganglion was chosen as an experimental model for this study because it is comprised of an homogeneous population of neurons, (the ciliary and the choroids neurons), which undergo well-synchronized developmental steps representing the different phases of synapse formation, and therefore it is amenable for a detailed characterization of gene expression profile in a postsynaptic element of a neuron-neuron synapse during synapse development. A detailed analysis of the onset and development of synaptic contacts within the ciliary ganglion has been carried out at the physiological and structural level (Landmesser and Pilar, 1978). The ciliary ganglion precursor cells migrate out of the cranial neural crest at embryonic day (E) 2.5 (St 9), and reach their final destination at E4 (St 24–25) (Hammond and Yntema, 1958; Landmesser and Pilar, 1978). By E5 (St 26), the ganglion is anatomically distinct, and neurons are electrically excitable. The preganglionic afferent fibers from the accessory oculomotor Edinger-Westphal nucleus located in the mesencephalon reach the ciliary ganglion at E5; however, no synaptic transmission through the ganglion is detected at this stage (Landmesser and Pilar, 1976). At E6 (St 26–27), a small number of neurons become depolarized by stimulating the presynaptic fibers, indicating the onset of synapse formation. During the next 2–3 days, bouton-like synaptic contacts develop between the preganglionic terminals and the ciliary ganglion neurons, and by E8 (St 33) synaptic transmission can be triggered in all ganglionic neurons by presynaptic stimulation. After a brief period of polyneuronal innervation, the bouton-like terminals formed on the ciliary neurons are progressively replaced by a calyx-type monosynaptic contact. Between E9 and E12 (St 35 to St 38), the calyciform presynaptic terminal differentiates, forming multiple active zones on the ciliary neuron body, and by E17 (St 43) the synaptic complexes have become morphologically and physiologically matured (Landmesser and Pilar, 1972; Landmesser and Pilar, 1978).

Analysis of gene expression profiles during synapse development in the ciliary ganglion

To identify gene transcripts expressed during the different phases of synapse development a genome-wide analysis of gene expression profiles in the developing ciliary ganglion was carried out using the GeneChip® Chicken Genome Array from Affymetrix. This microarray contains 38,535 probes providing comprehensive coverage of 32,773 transcripts corresponding to 28,416 predicted chicken genes from the Ensembl annotation of the complete chicken genome. Microarray analysis was performed using RNA isolated from ciliary ganglia at four different developmental stages representing different phases of synapse formation: E5 (St 26) absence of synaptic contacts, E8 (St 33) initiation of synapse formation, E10 (St 36) synapse differentiation, and E17 (St 43) synapse maturation. The microarray analysis of each developmental stage was carried out in triplicates and each replicate was done with independent tissue collection and RNA extraction. A gene-transcript was considered present when its expression levels were above background in at least two out of the three GeneChips analyzed at each developmental stage with a detection p-value <0.05

(Student's T-test). This analysis detected the expression of 21,493 genes throughout development, which show substantial changes in gene expression levels associated with each phase of synapse formation (Fig 1, Table 1).

To determine which gene transcripts are associated with each phase of synapse development, the level of gene expression at each stage was first compared with the expression level at E5. A gene was considered up or down-regulated when its expression levels varied by ≥ 2 -folds with a detection p-value < 0.05 (Student's T-test). These comparisons revealed that 469 gene-probes were up-regulated between E5 and E8, 826 probes between E5 and E10, and 1974 probes between E5 and E17 (Table 1). Similarly, a substantial decrease in gene expression levels was observed at each developmental stage, including 367 probes down-regulated between E5 and E8, 435 probes between E5 and E10, and 1,203 probes between E5 and E17 (Table 1).

To identify genes whose expression co-relates with each phase of synapse development, the level of expression of each gene was compared between consecutive developmental stages. These comparisons showed that at E8, 469 gene probes were ≥ 2 -fold up-regulated as compared to their expression levels at E5, 67 gene probes were up-regulated between E8 and E10, and that 1,200 gene probes were up-regulated at E17 as compared to E10. Similarly, 367 genes were ≥ 2 -fold down-regulated between E5 and E8, 13 genes between E8 and E10, and 446 genes between E10 and E17 (Table 1). This analysis shows that the number of up and down regulated genes increases with development (Fig 1, Table 1). Changes in gene expression levels are particularly pronounced between E10 and E17 probably because this period of development involves multiple cellular functions including synaptic maturation within the ciliary ganglion, myelination of cell bodies and axons, natural occurring neuronal death, and differentiation of neuromuscular terminals in the target tissue. Thus, the association between gene expression levels and a particular cellular function becomes more difficult at later stages of development. In contrast, the number of genes associated with the period of initiation of synapse formation (E5 to E8) is more limited. This is presumably due to the fact that neuronal migration and differentiation have largely ended by E5, and that the initiation and differentiation of synaptic contacts are the primary cellular events that take place between E5 and E8.

Identification of gene transcripts associated with the initiation of synapse formation

The initial phase of synapse formation in the ciliary ganglion starts at E6 and progresses until E8. At E5 the presynaptic fibers are in the vicinity of the ganglionic neurons; however, synaptic connections have not yet developed, suggesting that at E5 the proteins involved in the induction of presynaptic differentiation are absent or expressed at insufficient amounts. In contrast, by E8 all ganglionic neurons have developed functional synapses (Landmesser and Pilar, 1978). Thus, the 469 gene-probes ≥ 2 -fold up-regulated between E5 and E8 constitute a pool genes likely to encode proteins with synaptogenic activity.

To sort out which probes from this pool represent a gene encoding suitable synaptogenic proteins, each probe was individually analyzed to determine whether it detected a gene encoding a known or predicted protein, a hypothetical protein, or an expression sequence tag (EST). If a gene was detected by more than one probe, the probe that gave the highest fold increase was considered for further analysis. This analysis showed that from the 469 probes up-regulated between E5 and E8, 302 probes detected a transcript encoding a known or predicted protein with characterized protein domains.

The induction of presynaptic differentiation by the postsynaptic cell requires the expression of synaptogenic proteins on the cell surface or its secretion into the extracellular space. Therefore, the 302 gene transcripts were analyzed to determine whether they encoded a

protein containing a signal peptide for secretion, a putative transmembrane domain, or a glycosylphosphatidyl inositol (GPI) link sequence. To this end, the 302 transcripts were sorted out into three main groups: a) intracellular (185 transcripts), b) cell membrane bound (93 transcripts), and c) secreted (24 transcripts). Proteins with a transmembrane domain, which are associated with intracellular organelles (ER/Golgi, mitochondria, or nucleus) were considered intracellular. Finally, as postsynaptic proteins capable of inducing presynaptic differentiation should operate as cell surface ligands, the protein domains of the 75 cell membrane associated gene transcripts up-regulated at E8 were examined and sorted out according to their predicted function. This examination identified 26 proteins which can potentially operate as cell surface ligands, 20 signaling receptors (kinases, G-protein coupled receptors, etc.), 29 transporters and ion channels, and 18 hypothetical proteins (Fig 2). Therefore, from the 469 probes ≥ 2 -fold up-regulated between E5 and E8, a pool of 51 protein-coding transcripts (24 secreted and 27 membrane bound) was identified which are potentially involved in the initiation of synapse formation (Table 2).

Analysis of the 27 cell membrane associated proteins identified 12 cell adhesion molecules including, seven immunoglobulin superfamily (IgSF) members, 3 members the cadherin superfamily, and 2 proteins which have been associated with cell adhesion in non-neuronal cells. Six other proteins are known to operate as ligands or as ligand/receptors, and 6 proteins can potentially operate as membrane bound ligands but do not belong to any particular class of proteins. In addition, 3 membrane-bound proteins possessing enzyme-like domains were identified. Finally, 24 secreted proteins could be sorted into 5 groups including, 8 components of the extracellular matrix (ECM), 3 neuropeptides, 3 cytokines and growth factors, 5 modulators of secreted ligands, and 5 secreted proteins with unrelated domains (Table 2).

The expression profile throughout development of the transcripts described in Table 2 was examined to identify the gene transcripts up-regulated at E8 which are primarily associated with the first phase of synapse formation. To this end the average signal intensity at each developmental stage analyzed was normalized to their levels at E5 (E5 signal intensity = 1) and plotted in a $\log_2$ scale against embryonic age (Figure 3). This analysis showed that gene expression levels follow three main profiles. Genes that continue to up-regulate after the initiation of synapse formation, genes with constant expression levels until the end of embryonic development, and genes which are down-regulated after E8. A gene was considered up-regulated when its average signal was increased between E8 and E17 with a p-value < 0.05 (T-test). This analysis showed that 36% of the gene transcripts presented in Table 2 was further up-regulated between E8 and E17. These genes include: IgSF, IGSF11 and VCAM; Cadherin superfamily, PCDH9, CDH13, and CDH5; Other CAMs, CD99; Ligand/Receptors, neurexin-4, LRRTM3, and DLK1; Other Ligands, LGL1 and PSCA; Enzymes, P. Phosphatase; Neuropeptides, SS2 and PNOC; Cytokines, IGFBP2 and GAS6; ECM, COL14A1 and COL3A1; and Modulators of secreted factors, Sulfi-1. Thus, 64% of the 51 selected gene transcripts are not substantially up-regulated after E8.

Effect of synaptic activity blockade on gene expression levels of presumptive synaptogenic proteins

Electrical activity and synaptic transmission participate in various aspects of neural development including refinement of synaptic connections, cell survival (Katz and Shatz, 1996; Meriney et al., 1987; Zhang and Poo, 2001), and regulation of neurotransmitter receptors (Jacob and Berg, 1987). Although the balance between synapse formation and synaptic withdrawal is affected by synaptic activity, formation of synaptic complexes can proceed in the absence of synaptic transmission. For example, development of synaptic junctions and differentiation of synaptic terminals occurs in animals in which neurotransmitter release is blocked by genetic disruption of Munc-18 expression (Verhage et

al., 2000), or by pharmacological blockade of synaptic activity (Kaiser et al., 2002). Therefore, to examine the role of synaptic transmission on the expression levels of the genes up-regulated during the initial phase of synapse formation, chicken embryos were treated daily with d-tubocurarine (1mg/day) from E5 until E8. Tubocurarine is an acetylcholine receptor antagonist that blocks synaptic transmission through the ciliary ganglion (Meriney et al., 1987). To determine the effectiveness of d-tubocurarine treatment in blocking cholinergic transmission, the number of spontaneous hindlimb kicks was quantified. Chicken embryos start twitching at E6 by muscle contraction triggered by spontaneous motor neuronal stimulation, and this activity is blocked by d-tubocurarine (Meriney et al., 1987). At E8, the ciliary ganglia from embryos treated with d-tubocurarine and showing ≤ 3 kicks/min were collected and gene expression levels were examined by GeneChip analysis as described above. Untreated animals had an average of 16.6 ± 0.86 S.E.M. kicks/min.

The effect of synaptic activity blockade on the overall levels of gene expression was first examined by comparing the number of genes present in control embryos at E8 (18,819) with the genes present in the samples obtained from d-tubocurarine treated embryos (18,450). A Venn diagram from the two groups shows that the majority (89%) of the genes expressed in control samples were also present in the samples treated with d-tubocurarine, indicating that the pharmacological treatment did not substantially affect gene expression during embryonic development (Fig 4A). Next, the expression levels of the genes that were ≥ 2 -fold up-regulated between E5 and E8 during the initiation of synapse formation were examined. The comparison shows that 48% (228 out of 469) of the genes up-regulated in control animals were also up-regulated in the d-tubocurarine treated embryos, while 727 new probes were up-regulated by the treatment with d-tubocurarine (Fig 4B). Similarly, 58% (212 out of 367) of the gene-probes ≥ 2 -fold down-regulated in control embryos were also down-regulated in d-tubocurarine treated samples. In contrast, 2,346 new gene-probes were ≥ 2 -fold down-regulated by activity blockade (Fig 4C), indicating that synaptic activity is an important driving force for gene expression.

To determine whether the expression levels of the genes up-regulated between E5 and E8 (Table 2, Fig 3) were affected by synaptic activity, the expression levels between E8 control and d-tubocurarine treated samples were examined. The expression levels of 18 genes out of the 51 genes included in Table 2 were affected by synaptic activity blocked (T-test $p < 0.05$). The average signal of 6 gene transcripts was significantly up-regulated, while the expression levels of 12 genes was reduced as compared to their values at E8. Analysis of the effect of d-tubocurarine on the expression levels of cell adhesion molecules shows that members of the IgSF including contactin 5, DSCAM, and LSAMP, were partially enhanced by synaptic activity blockade, while all the other members of the IgSF were not substantially affected (Table 3). In contrast, the expression levels of CD99L2, the surface ligands SLITRK1, LRRTM3, attractin 1, GSGL1, and two proteins with enzymatic activity, MMEL1 and GPD4, were reduced by treatment with d-tubocurarine. The effect of d-tubocurarine on the expression levels of genes encoding proteins of the ECM was also divided. While the expression levels of reelin and olfactomedin-2B were increased, the expression of EDIL3 and collagen 3A1 was reduced by treatment with d-tubocurarine. Expression levels of the neuropeptide nociceptin, the modulator of secreted factors DKK3, and the unrelated secreted protein CAPRN2 were also reduced by d-tubocurarine treatment.

Discussion

Synapse formation is a complex and dynamic process involving a variety of mechanisms carried out by diverse groups of molecules. The initiation of the formation of a synapse requires molecular interaction between presumptive synaptic membranes that induce the differentiation of pre and post synaptic compartments (Akins and Biederer, 2006;

McAllister, 2007). The present study carried out a genome-wide analysis of gene expression levels during synapse formation with the aim of identifying gene transcripts expressed by postsynaptic neurons encoding proteins presumably capable of inducing presynaptic differentiation. Two main criteria were used for the selection of gene transcripts namely, the induction of gene expression during the initiation of synapse formation, and the ability of the encoded protein to operate as membrane bound or secreted ligand. In addition, the expression levels of gene transcripts encoding cell surface proteins up-regulated between E5 and E8 were assessed under pharmacological blockade of synaptic transmission.

The initiation of synapse formation in the ciliary ganglion occurs between E5 and E8. However, synapse formation is not the only event taken place during this developmental period, in that axons from the ciliary ganglion neurons are also interacting with their target tissues in the internal muscle of the eye. In addition, the ciliary ganglion contains glial cells that start the myelination process right after synapses are formed. Therefore, it is possible that the gene-transcripts identify in this study are not exclusively involved in the initiation of synapse formation, and they may also participate in other cellular processes. However, the criteria used in this study to select gene-transcripts were aimed at identifying proteins whose developmental pattern of expression and molecular features indicates presumptive synaptogenic activity. This selection of gene transcripts identified a group of proteins which are primarily expressed by neurons, and some of which are affected by synaptic activity suggesting that these proteins contribute to the development of synaptic contacts.

Cell adhesion molecules

CAMs of the IgSF engage in homo and/or heterophilic interactions involved in cellular recognition and adhesion. CAMs contribute to a variety of neurodevelopmental processes including neurite outgrowth, axonal pathfinding, and synapse formation (Rougon and Hobert, 2003). Gene transcripts encoding several proteins of the IgSF were detected during ciliary ganglion development including, NCAM, Ng-CAM/L1, nectin, synCAM/IGSF4, DSCAM, contactin-1, and contactin-5. However, no significant changes in gene expression levels of NCAM, nectin, Ng-CAM/L1, and synCAM were found during the initiation of synapse formation (data not shown). Although synCAM is sufficient to induce presynaptic differentiation (Biederer et al., 2002), it appears to be dispensable for the initiation of synapse formation in the ciliary ganglion, where it contributes to synapse maturation (Triana-Baltzer et al., 2008). Therefore, the expression profile of synCAM indentified in this study is in agreement with the previous analysis of synCAM function in the ciliary ganglion (Triana-Baltzer et al., 2008). Nevertheless, synCAM is capable of inducing the formation of glutamatergic synapses (Biederer et al., 2002), suggesting that different neurons or synaptic types may use distinct synaptogenic mechanisms.

Three related members of the IgSF, contactin-1, contactin-5, and DSCAM, showed an expression profile that correlates with the initiation of synapse formation. DSCAM constitute a family of proteins which by alternative splicing generates a large number of isoforms exerting specific homophilic binding (Wojtowicz et al., 2004). Previous studies have found that DSCAM and sidekicks mediate intercellular recognition necessary for synapse formation in specific cellular layers (Hattori et al., 2008; Yamagata and Sanes, 2008). Therefore, it is not surprising that DSCAM is up-regulated precisely during the initiation of synapse formation in the ciliary ganglion. In addition, this study also found that DSCAM expression levels were increased by pharmacological blockade of synaptic transmission, suggesting that DSCAM may contribute to structural changes associated with activity-dependent synaptic plasticity.

Contactins are a subfamily of the IgSF of proteins characterized by an ectodomain containing six immunoglobulin domains and four fibronectin type III repeats, which is

anchored to the plasma membrane by a GPI moiety (Ranscht, 1988). Contactin-1 is localized at synaptic junctions and its expression is restricted to one side of the synapse, suggesting that it may participate in synapse formation (Faivre-Sarrailh et al., 1992). Genetic deletion of contactin-1 (CNTN1) results in severe ataxia due to defective axonal guidance and dendritic projections of cerebellar granule cells (Berglund et al., 1999). Moreover, CNTN1 mutations in humans have been linked to autism spectrum disorders (Compton et al., 2008), underscoring the importance of this subfamily of proteins during the final steps of the assembly of the neural circuits. However, whether contactins are directly involved in the initiation of synapse formation has not been investigated. Thus, the expression of contactin 1 and 5 during the initiation of synapse formation, and their resilient expression under synaptic activity blockade, suggest that these two molecules could be important factors during the induction of the synapse formation.

Two other IgSF proteins, kirrel-3 and LAMP, were found to be highly and specifically expressed during the initiation phase of synapse formation. Kirrel-3 contains five immunoglobulin domains in its extracellular portion, a single transmembrane domain, and a PSD-95, Dlg and Zo-1 (PDZ) domain-binding motif at the C-terminus of the cytoplasmic tail (Ueno et al., 2003). In *C. elegans*, the kirrel-3 homology SYG-1, determines the localization of synapse formation and its expression is sufficient to trigger the accumulation of synaptic vesicles (Shen and Bargmann, 2003). In the mouse, kirrel-3 is expressed in various regions of the developing and adult nervous system including the olfactory bulb, where it contributes to the selection of appropriate synaptic targets (Serizawa et al., 2006), suggesting that kirrel-3 possess synaptogenic activity (Gerke et al., 2006). Similarly, LAMP is an IgSF member containing three immunoglobulin domains bound to the cell membrane by a GPI anchor (Pimenta et al., 1996). LAMP is expressed by subsets of cortical and sub-cortical neurons in the limbic region of the brain (Horton and Levitt, 1988), and disruption of LAMP expression or function causes abnormal mossy fiber growth into the hippocampus and behavioral deficits (Catania et al., 2008). However, whether LAMP contributes to synapse development has not been investigated.

Cadherins and protocadherins are localized at synaptic junctions from the onset of synapse formation and they have been implicated in various aspects of synapses development (Takeichi, 2007; Morishita and Yagi, 2007). N-cadherin, contributes to dendritic spine development and synaptic maturation (Bozdagi et al., 2004; Brusés, 2000; Togashi et al., 2002); however, a distinct role for N-cadherin in the initiation of synapse formation has not been demonstrated yet. In this study, three members of the cadherin superfamily (T-cadherin, VE-cadherin, and protocadherin 9) were found to be up-regulated during the initiation of synapse formation and their expression levels continue to increase throughout development, suggesting that cadherins participate in the assembly and maintenance of the synaptic junction but they may be dispensable for the induction of synapse formation (Bozdagi et al., 2004; Kadowaki et al., 2007; Scheiffele et al., 2000).

Cell surface ligand/receptors

SLITRK-1 and Eph-A5 are the only two members of this group that showed an expression profile that correlates with the first phase of synapse formation. SLITRKs are a family of single-pass transmembrane proteins characterized by leucine rich repeats (LRR) in the extracellular domain, which are similar to the LRR domains found in the Slit family of proteins (Aruga and Mikoshiba, 2003). Sequence variations in SLITRK-1 have been found in individuals with Tourette's syndrome (Abelson et al., 2005). These mutations cause a frame-shift that introduces a stop codon in the extracellular domain causing a premature termination of translation which is likely to results in the loss of protein expression, suggesting that SLITRK-1 may participate in the induction or assembly of synaptic contacts. Although the receptor for SLITRK-1 has not been identified, it may operate as a

postsynaptic ligand for a presynaptic receptor. LRRTM3 belongs to a recently identified small family of genes encoding LRR transmembrane proteins highly conserved among vertebrates (Lauren et al., 2003), possess a potential PDZ binding motif at the C-terminal end, and is predominantly expressed in neurons of the developing nervous system, suggesting that it may play a role in the organization of the postsynaptic apparatus.

Ephs are a family of receptor tyrosine kinases that are divided into A and B subclasses based on their affinity for the ephrin-A or ephrin-B ligands (Gale et al., 1996). Both, Ephs and ephrins can originate bidirectional signaling to control cell-cell communication. The majority of the studies on the role of ephrins in synapse formation have focused on the Eph-B/ephrin-B subfamily. This subfamily of Eph-ephrins participates in axonal guidance and localizes at synaptic contacts where they contribute to spine formation, presynaptic differentiation, and clustering of glutamate receptors (Dalva et al., 2000). Eph-A subfamily of receptors is also localized at the dendrites of hippocampal neurons and regulate spine morphology (Murai et al., 2003), suggesting that it contribute to postsynaptic differentiation. However, Ephrin-A are GPI-linked to the membrane and by themselves cannot initiate signaling, suggesting that Eph-A5 in the ciliary ganglion is primarily involved in postsynaptic differentiation.

Neuroligins are expressed by postsynaptic cells and their binding with presynaptic neuroligins is sufficient to induce presynaptic differentiation (Scheiffele et al., 2000). However, the precise role that neuroligin and neuroligins play in synapse formation is not completely understood in that deletion of the three neuroligins isoforms does not preclude the initiation of synapse development, but instead results in defective synapse maturation (Varoqueaux et al., 2006). In the ciliary ganglion neurons both neuroligins and neuroligins are expressed throughout development (data not shown); however, no specific correlation in the levels of expression was found with any phase of synapse formation, except for neuroligin-4 which was highly up-regulated during the initiation of synapse formation. In addition to their role in presynaptic differentiation, neuroligins participate in the assembly of postsynaptic components (Graf et al., 2004), suggesting that neuroligin-4 contribute to postsynaptic differentiation in ciliary ganglion.

Cell surface ligand-like proteins

Connexin-36 is a neuron-specific gap-junction protein that participates in electrical coupling between neurons, and is widely expressed in the CNS of vertebrates (Condorelli et al., 1998). Gap junctions play an important role in neuronal coupling during development (Kandler and Katz, 1995). Intercellular coupling through gap junction is widespread during synapse formation and the initial establishment of neuronal circuits, suggesting that gap junctions may provide interneuronal communication that contributes to the generation of synaptic contacts (Kandler and Katz, 1995; Personius et al., 2001). Furthermore, connexins expressed by apposed cell membranes form adhesive structures that may promote the development of adhesive junction associated with chemical synaptic contacts.

The NYX gene encodes the protein nyctalopin which belongs to a small family of a cell surface proteoglycan containing eleven LRRs in the ectodomain attached to the membrane by a GPI-link (Bech-Hansen et al., 2000). NYX is mutated in congenital stationary nightblindness, a retinal disorder characterized by impaired night vision and myopia. Nyctalopin is localized to postsynaptic terminals in the retina and its deletion causes impaired synaptic transmission, suggesting that nyctalopin might be involved in certain aspects of synapse development (Bahadori et al., 2006; Bech-Hansen et al., 2000). However, the precise role of NYX in synapse formation and/or synaptic function has not been elucidated. Nevertheless, other members of this family of LRR proteoglycans have important roles as cell adhesion molecules during neural development (Krantz and Zipursky,

1990) suggesting that nyctalopin can potentially operate as a cell adhesion receptor supporting synapse formation.

Extracellular matrix components and secreted proteins

Various ECM components were found to be induced during the initiation of synapse formation. From this group, reelin, KAL-1, and olfactomedin have been shown to participate in neuronal development (Borrell et al., 1999; Loria et al., 2004; Rugarli et al., 1993). Reelin participates in neuronal migration, dendritic arborization and synaptic transmission (D'Arcangelo, 2006; Herz and Chen, 2006). In addition, reelin participates in the stabilization of dendritic spines (Niu et al., 2008) and the maturation of neuromuscular junctions (Quattrocchi et al., 2003). Reelin is produced and secreted by motor neurons and animals lacking reelin show defective postsynaptic differentiation and increased number of synaptic vesicles in the presynaptic terminal. Ciliary ganglion neurons innervate the internal muscles of the eye and therefore, it is possible that reelin expression is related to a role at the neuromuscular terminal. However, the peak of reelin expression occurs before the development of the neuromuscular junctions, suggesting that reelin could also participate in presynaptic development within the ciliary ganglion.

KAL-1 is the gene underlying the X-chromosome linked form of the Kallmann syndrome, which is characterized by anosmia and hypogonadism (Naftolin et al., 1971). KAL-1 encodes anosmin-1 which is present in various extracellular matrices during organogenesis, including the olfactory tract, the olfactory cortex, and operates as a chemo-attractant (Soussi-Yanicostas et al., 2002). Anosmin-1 contains an N-terminal cysteine rich repeat, a whey acidic protein domain and four fibronectin type III repeats similar to the ones found in the IgSF member axonin-I/TAG-1/CNTN-2. Anosmin-1 promotes intercellular adhesion and missense mutations within the fibronectin repeats found in individuals with Kallman syndrome result in an increase in cellular adhesion (Robertson et al., 2001; Soussi-Yanicostas et al., 1998). Fibronectin repeats do not directly contribute to adhesive interactions; however, *cis* interactions between adjacent molecules and with immunoglobulin domains within the same molecule regulate the adhesive properties of IgSF CAMs (Freigang et al., 2000; Kunz et al., 2002). Therefore anosmin-1 could be necessary for presynaptic differentiation by regulating adhesive interactions between CAMs within the synaptic cleft.

Olfactomedins are a large group of ECM glycoproteins characterized by the presence of an olfactomedin domain in their C-terminal region (Karavanich and Anholt, 1998). Most of the members of this family are expressed in neural tissue and they contribute to various developmental processes including neurogenesis, neuromuscular signaling, and synaptic vesicle exocytosis (Barembaum et al., 2000; Loria et al., 2004). The mechanism of action of olfactomedins is mostly unknown. However, expression of *unc-22*, a transmembrane protein containing an olfactomedin domain, is required for maintaining a structural microenvironment necessary for proper synaptic function in the *C. elegans* neuromuscular junction (Loria et al., 2004). Therefore, olfactomedin-2B may contribute to the organization of the extracellular environment necessary for the presentation of cell surface ligands involved in presynaptic terminal differentiation.

Three other groups of gene transcripts encoding secreted proteins and peptides were found expressed during the initiation of synapse formation and their expression was not affected by the inhibition of synaptic transmission. These include, the GnIH, IL-9, TNF-1, WIF-1 and DKK-3. Neuropeptides and neurotransmitters can regulate synapse development but they are usually released presynaptically and regulate postsynaptic differentiation. Cytokines and growth factors affect neuronal differentiation and synapse formation (Kim et al., 2002; Mehler et al., 1993). Whether IL-9 and TNF-1 possess synaptogenic activity has not been

investigated; however, molecules originally thought as being exclusive component of the immune system have been found expressed in neurons and playing important roles in regulating synaptic plasticity (Corriveau et al., 1998). Therefore, these two cytokines may directly bind and signal through presynaptic receptors, or regulate cell surface expression levels of other relevant receptors involved in synaptogenesis.

The WNT family of secreted factors has been implicated in axonal pathfinding and synapse formation (Ciani and Salinas, 2005). WNTs operate as a target-derived signal that controls the size and shape of the presynaptic terminal and the maturation of synaptic contacts (Ciani and Salinas, 2005). The activity of WNTs is regulated by extracellular modifiers, including WNT inhibitory factor-1 (WIF1), the soluble frizzled-like proteins (sFRP), dickkopf (Dkk), and sulfatase 1 (sulfi1) (Kawano and Kypta, 2003). Analysis of WNT expression during ciliary ganglion development revealed that from the eleven WNTs identified in the chicken genome, only *wnt5a* and *wnt6* are expressed in the ciliary ganglion; however, both of them are undetectable or expressed at low levels during the initiation of synapse formation, and become up-regulated during synaptic maturation (data not shown). In contrast, WIF-1, Dkk-2, and Dkk-3 expression is up-regulated during first phase of synapse formation and down-regulated at later phases, suggesting that WIF-1 and Dkk may have activities and functions independent of WNT. Furthermore, the WIF domain of WIF-1 is a protein interacting domain related to the immunoglobulin fold (Patthy, 2000) that may regulate cell signaling by direct interaction with cell surface receptors. However, its role during neural development and its mechanism of action remains largely uncharacterized.

Finally, a group of unrelated secreted proteins including cathelicidin, neuroserpin, PD-L1, and cerebellin-4 were found up-regulated during the first phase of synapse development. These proteins are secreted ligands that affect diverse cellular functions in the nervous and immune systems, but none of them has been studied during synapse development.

In summary, the present study identified a group of protein coding gene transcripts, which according to their expression profile and molecular features are candidates of synaptogenic proteins. Two main groups of proteins were identified, cell adhesion molecules and components of the extracellular matrix. This is not surprising as various proteins from these families participate in the assembly of synaptic contacts. However, the IgSF members contactins, kirrel-3, and LAMP, and the ECM components KAL-1, olfactomedin, and reelin, were identified as likely candidates of proteins that contribute to the initiation of synapse development. In addition, various surface proteins containing protein interacting domains including the LRR motif were identified as potentially synaptogenic proteins. Thus, this study provides a comprehensive view of the potential synaptogenic molecules expressed by postsynaptic neurons, which facilitates the investigation of cooperative and/or synergistic molecular mechanisms underlying the induction of synapse formation.

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Figure 1.

Gene expression profile during the different phases of synapse development in the chick ciliary ganglion. Ciliary ganglia were isolated at each of the four developmental stages depicted in the graph (E5, E8, E10, and E17) and gene-transcript levels were assessed with the chicken GeneChip microarray from Affymetrix. The normalized signal intensity of each probe is plotted on a $\log_{10}$ scale. From the 38,535 probes present in the microarray 21,493 are plotted, which represent the set of probes present in at least one developmental stage. Note the substantial up and down-regulation of gene expression levels during initiation (E5 to E8) and maturation (E10 to E17) of synapse formation.

Figure 2.

Distribution of the 302 protein coding gene transcripts ≥ 2 -fold up-regulated during the initiation of synapse formation (E5 to E8) according to their subcellular localization and protein function. The protein coded by each gene transcript was examined and sorted into one of six groups according to its molecular features, 1, secreted (24); 2, cell surface ligands (27); 3, cell surface receptors (20); 4, ion channels and solute carriers (29); 5, proteins with uncharacterized domains (18); 6, intracellular proteins (185).

Figure 3.

Expression profile throughout embryonic development of the gene transcripts described in Table 2. Expression levels of all transcripts at E5 were normalized to 1. The plots depict the expression levels of each gene transcript at each developmental stage as compared to their levels at E5. The Y axis is in $\log_2$ scale for convenience. A) Members of the IgSF, B) Members of the cadherin superfamily, C) Other CAMs, D) Cell surface ligands-receptors, E) Cell surface proteins that can potentially operate as ligands, F) Cell surface proteins with enzyme-like domains, G) Components of the extracellular matrix, H) Neuropeptides, I) Cytokines and trophic factors, J) Proteins that modulate the activity of secreted signaling molecules, K) Secreted molecules with unrelated protein interacting domains.

Figure 4.

Venn diagrams illustrating the number of genes whose expression was affected by synaptic activity blockade. A) Comparison between all gene transcripts expressed at E8 control with the ones expressed at E8 treated with d-tubocurarine. B) Comparison between the groups of gene transcripts that were ≥ 2 -fold up-regulated during the initiation of synapse formation (E5 to E8, T-test p-value < 0.05) under control and synaptic activity blockade. C) Comparison between the groups of gene transcripts that were ≥ 2 -fold down-regulated during the initiation of synapse formation (E5 to E8, T-test p-value < 0.05) under control and synaptic activity blockade.

Table 1

Changes in gene expression levels during embryonic development in the CG

Number of gene-probes present in the ciliary ganglion at each developmental stage. A probe was considered present when the signal value was above background with a p-value <0.05 in at least two out of the three gene chips analyzed at each developmental stage. The number of probes with ≥ 2 -fold change between stages and p-value <0.05 are shown.

	E5 (St 26)	E8 (St 32)	E10 (St 36)	E17 (St43)
Number of genes expressed at each developmental stage	18767	18819	19247	19154
Number of genes 2-fold up-regulated vs E5		469	826	1974
Number of genes 2-fold up-regulated between consecutive stages		469 (vs E5)	67 (vs E8)	1200 (vs E10)
Number of genes 2-fold down-regulated vs E5		367	435	1203
Number of genes 2-fold down-regulated between consecutive stages		367 (vs E5)	13 (vs E8)	446 (vs E10)

Table 2

Gene transcripts up-regulated during the initiation of synapse formation
List of gene transcripts ≥ 2 -fold up-regulated during the initiation of synapse formation (E5 to E8, T-test p-value < 0.05) encoding proteins with signal peptide for secretion or putative transmembrane domains.

	Gene Symbol	Affymetrix Probe	Description	Fold increase E8 vs E5	Expressed by neurons
1) Cell Surface Membrane Proteins					
a) Cell Adhesion Molecules					
Ig superfamily member 11 isoform a	IGSF11	Gga.1594.1.S1_at	Ig superfamily. Coxsackie virus and adenovirus receptor	6.83	Yes
Contactin 1	CNTN1	Gga.4796.1.S1_at	Ig superfamily. F3/FI1. GPI-linked	3.45	Yes
Contactin 5	CNTN5	Gga.945.1.S1_at	Ig superfamily. NB-2. GPI-linked	3.03	Yes
Down syndrome cell adhesion molecule	DSCAM	Gga.5827.1.S1_at	Ig superfamily	2.9	Yes
Kin of irregular chiasmprotein 3	LOC419719	GgaAffx.755.2.S1_s_at	Ig superfamily. Kirre-3	2.86	Yes
Vascular cell adhesion protein 1	VCAM1	Gga.12928.1.S1_at	Ig superfamily	2.31	Yes
Limbic system-associated membrane protein	LSAMP	Gga.4154.2.S1_a_at	Ig superfamily. LAMP. GPI-linked	2.79	Yes
Protocadherin 9	PCDH9	Gga.3348.1.S1_at	Cadherin superfamily	4.76	Yes
Cadherin 13	CDH13	Gga.3903.1.S1_at	T or H-cadherin. GPI-linked	2.77	Yes
Cadherin 5	CDH5	Gga.5171.1.S1_at	VE-cadherin	2.02	Yes
CD99 molecule 2 isoform E3-4	CD99L2	Gga.11915.1.S1_at	Cell adhesion like surface glycoprotein (MIC2L)	2.53	Yes
CD99 isoform E2	CD99	Gga.12625.1.S1_at	Cell adhesion likel surface glycoprotein (MIC2)	2.51	N/D
b) Ligand/Receptor					
SLIT and NTRK-1	SLITRK1	Gga.14205.1.S1_s_at	Mutated inTourette's syndrome	5.2	Yes
Ephrin type-A receptor 5	EPHA5	Gga.3047.2.S1_at	Eph ligand	5.1	Yes
Neurexin 4	LOC428309	Gga.6098.1.S1_at	Silimilar to contactin	4.64	Yes
LRR-transmembrane neuronal protein 3	LRRTM3	Gga.16173.1.S1_at	Leucine rich transmembrane nerual protein	3.90	Yes
Growth arrest-specific protein	PMP22	Gga.4856.1.S1_s_at	Peripheral Myelin protein 22/tight junction	3.17	Yes
Delta-like 1 homolog	DLK1	Gga.9975.1.S1_at	Notch ligand	2.02	Yes
c) Cell Surface Ligand-like Proteins					
Connexin 36	GJD2	Gga.96.2.S1_at	Gap-junction protein delta	5.05	Yes
DD1 protein	DD1	GgaAffx.4057.1.S1_at	Uncharacterized protein family	3.26	N/D
Attractin 1	ATRNL1	GgaAffx.5785.2.S1_s_at	Binds to MC4-R	2.74	Yes

	Gene Symbol	Affymetrix Probe	Description	Fold increase E8 vs E5	Expressed by neurons
d) Enzymes	Germ cell associated 1	GgaAffx.8377.1.S1_at	Uncharacterized protein family	2.67	N/D
	Nyctalopin	GgaAffx.10366.1.S1_at	Congenital stationary night blindness-1.GPI-linked	2.14	Yes
	Prostate stem cell antigen	Gga.6070.1.S1_at	Thy-1/Ly6 family. UPAR/Ly6 domain. GPI-linked	2.12	N/D
	Metallo-endoropeptidase-2	GgaAffx.751.1.S1_s_at	Endopeptidase with unknown cellular function	5.12	N/D
	GDPD4	GgaAffx.434.1.S1_at	Glycerophosphodiester phosphodiesterase domain	3.1	N/D
	Tyrosine specific protein phosphatase	GgaAffx.367.1.S1_at	Protein Phosphatase. Unknown cellular function	2.08	N/D
2) Secreted Proteins and Polypeptides					
a) Extracellular Matrix Proteins/Secreted Ligands					
Epidermal growth factor-like domain protein 6	EGFL6	Gga.11105.1.S1_a_at	ECM component. Nephronectin. Integrin ligand	6.33	N/D
	RELN	Gga.496.1.S1_at	ECM component	5.76	Yes
	KAL1	Gga.796.1.S1_at	ECM component. Mutated in the Kallmann Syndrome	5.54	Yes
	COL14A1	Gga.2558.3.S1_a_at	ECM component	4.61	Yes
	OLFML2B	GgaAffx.20643.1.S1_at	ECM component	3.46	Yes
Leucine-rich-glioma inactivated 1	LGI1	Gga.2756.1.S1_at	Mutated in autosomal dominant partial epilepsy	2.99	Yes
	EDIL3 (Del1)	Gga.18123.1.S1_at	Cell adhesion like molecule. Integrin ligand	2.81	N/D
	COL3A1	Gga.16844.2.S1_s_at	ECM component	2.05	Yes
b) Neuropeptides					
Somatostatin II	SS2	Gga.9088.1.S1_at	Modulator of synaptic transmission	52.85	Yes
GnIH	NPVF	Gga.9285.1.S1_at	Gonadotrophin inhibitory hormone/Neuropeptide VF	5.5	Yes
Noiceptin	PNOC	Gga.10041.1.S1_a_at	Modulator of synaptic transmission	2.23	Yes
c) Cytokines and Growth Factors					
Interleukin-9	IL9	Gga.11039.1.S1_at	Cytokine. Immune cell proliferation	2.78	N/D
Growth-arrest-specific protein 6	GAS6	Gga.8078.1.S1_at	Ligand for Axl and Rse/Tyrr 3 receptor protein tyrosine kinase	2.6	Yes
Tumor necrosis factor superfamily 13b	TNFSF13B	GgaAffx.13138.1.S1_at	Trophic factor	2.39	Yes
d) Modulators of Secreted Ligands					
Wnt inhibitory factor 1	WIF1	Gga.11611.1.S1_at	Regulator of wnt and shh signaling	12.77	Yes
Sulf1	LOC426312	Gga.18351.1.A1_at	Extracellular sulfatase. Regulator of wnt signaling	4.17	N/D

	Gene Symbol	Affymetrix Probe	Description	Fold increase E8 vs E5	Expressed by neurons
Insulin-like growth factor binding protein 2	IGFBP2	Gga.759.1.S1_at	Modulator of Insulin growth factor. Secreted ligand	2.68	N/D
Dickkopf-2	DKK2	GgaAffx.6667.1.S1_at	Regulator of wnt signaling	2.45	N/D
Dickkopf-3	DKK3	Gga.3573.2.S1_a_at	Regulator of wnt signaling	2.45	N/D
e) Unrelated Secreted Proteins					
Cathelicidin	CAMP	GgaAffx.21849.1.S1_s_at	Antimicrobial peptide	3.23	Yes
Neuroserpin	SERPINI1	Gga.4416.1.S1_at	Axonal secreted endopeptidase inhibitor	2.42	Yes
Carpin family member 2	CAPRIN2	Gga.10086.1.S1_s_at	Clq domain containing 1 isoform 2	2.24	N/D
PD-L1	CD274	GgaAffx.24574.1.S1_at	Programmed cell death 1 ligand (B7-H1)	2.08	N/D
Cerebellin 4	CBLN4	GgaAffx.4848.1.S1_s_at	ECM component	2.01	Yes

N/D, not determined.

Table 3

Effect d-TC on the expression of gene transcripts up-regulated during the initiation of synapse formation
 Effect of synaptic activity blockade on expression levels of the gene transcripts up-regulated during the initiation of synapse formation encoding cell surface proteins. The average signal of each affected gene and the percentage change as compared to control values are presented (T-test E8 control vs E8 curare p-value <0.05).

Protein	Gene symbol	Average signal E8 Control ($\pm$ SEM)	Average signal E8 d-TC ($\pm$ SEM)	% E8 d-TC of E8 Control
<u>Cell Surface Membrane</u>				
Contactin 5	CNTN5	517.4 $\pm$ 56.8	725.0 $\pm$ 46.7	140.1
Down syndrome cell adhesion molecule	DSCAM	415.1 $\pm$ 44.0	623.1 $\pm$ 27.1	150.1
Limbic system-associated membrane protein	LSAMP	3475.0 $\pm$ 237.1	8329.9 $\pm$ 234.4	239.7
CD99 molecule-2 isoform E3-4	CD99L2	3780.6 $\pm$ 235.7	2002.3 $\pm$ 447.7	53.0
CD99 isoform E2	CD99	1859.9 $\pm$ 166.0	3253.7 $\pm$ 254.2	174.9
SLIT and NTRK-1	SLITRK1	381.0 $\pm$ 13.2	34.6 $\pm$ 13.6	9.1
LRR-transmembrane protein 3	LRRTM3	156.0 $\pm$ 6.5	156.0 $\pm$ 2.1	73.1
Attractin 1	ATRNL1	480.3 $\pm$ 56.4	200.8 $\pm$ 67.8	41.8
Germ cell associated 1	GSG1L	285.6 $\pm$ 20.6	97.4 $\pm$ 20.9	34.1
Metallo-endopeptidase-2	MMEL1	1646.5 $\pm$ 184.1	407.0 $\pm$ 55.9	24.7
Glycerophosphodiester phosphodiesterase	GDPD4	782.2 $\pm$ 49.3	519.4 $\pm$ 38.9	66.4
<u>Secreted</u>				
Reelin	RELN	6648.4 $\pm$ 451.9	13531.9 $\pm$ 1702.7	203.5
Olfactomedin-2B	OLFML2B	5380.8 $\pm$ 292.7	7815.5 $\pm$ 534.6	145.2
EGF - Discoidin-1 domains Protein 3	EDIL3	700.6 $\pm$ 78.0	82.1 $\pm$ 26.1	11.7
Collagen	COL3A1	734.0 $\pm$ 104.2	253.4 $\pm$ 26.1	34.5
Nociceptin	PNOC	670.4 $\pm$ 7.4	440.1 $\pm$ 38.7	65.6
Dickkopf-2	DKK2	1018.2 $\pm$ 96.6	365.9 $\pm$ 133.6	35.9
Carpin family member 2	CAPRIN2	293.5 $\pm$ 30.3	120.8 $\pm$ 22.2	41.2

d-TC, d-tubocurarine.