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Expression patterns of neurexin-1 and neuroligins in brain and retina of the chick embryo: Neuroligin-3 is absent in retina

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Abstract

Neuroligins (NLs) constitute a family of cell-surface proteins that interact with neurexins (beta-Nxs), another class of neuronal cell-surface proteins, one of each class functioning together in synapse formation. The localization of the various neurexins and neuroligins, however, has not yet been clarified in chicken. Therefore, we studied the expression patterns of neurexin-1 (Nx-1) and neuroligin-1 and -3 during embryonic development of the chick retina and brain by reverse-transcriptase polymerase chain reaction (RT-PCR) and in situ hybridization (ISH). While neurexin-1 increased continuously in both brain and retina, the expression of both neuroligins was more variable. As shown by ISH, Nx-1 is expressed in the inner half retina along with differentiation of ganglion and amacrine cells. Transcripts of NL-1 were detected as early as day 4 and increased with the maturation of the different brain regions. In different brain regions, NL-1 showed a different time regulation. Remarkably, neuroligin-3 was entirely absent in retina. This study indicates that synaptogenetic processes in brain and retina use different molecular machineries, whereby the neuroligins might represent the more distinctly regulated part of the neurexin–neuroligin complexes. Noticeably, NL-3 does not seem to be involved in the making of retinal synapses.

Keywords: Chicken; Inner plexiform layer; Neurexin; Neuroligin; Retina development; Synaptogenesis

Neural cell adhesion proteins are key regulators of synaptogenesis in the nervous system. The neuroligins (NLs) constitute a family of neuronal transmembrane proteins that together with beta-neurexins form trans-synaptic cell adhesion complexes modulating recognition processes between neurons and synapse formation [2,4,5,12]. In rodents, they are localized to synapses [13,16] and are implicated in the scaffolding of synaptic proteins. Neurexins are alternatively spliced, constituting a highly diverse family of cell surface receptors [15]. Together with the thousands of isoforms of neurexins, the three neuroligins are hypothesized to engage in a highly coordinated combinational specificity that allows a precise communication between cells during development [15]. The current study was designed to address the following specific questions: (1) Are neurexins and neuroligins expressed in chicken brain and retina? (2) How do they differ in both parts? (3) Is their expression pattern correlated with synaptogenetic events?

The chick retina and brain can serve as suitable models for studying this development because the periods of synaptogenesis in the brain and retina have been described, the retina has a particularly broad inner plexiform layer (IPL), and the defined embryologic stages are easily available. Chicken embryos at different embryonic stages were isolated from eggs and decapitated, the eyes were enucleated and the brain was isolated. The telencephalon, optic tectum and the hindbrain were isolated starting with embryonic day 4 (E4). At E4 the tectum preparation represents actually the entire mesencephalon. Day 9 was the first stage that included cerebellum in the hindbrain preparation (see Fig. 1D). Following two wash steps in phosphate-buffered saline for 5 min, the eyes were either used for RNA isolation (TRI-REAGENT, Sigma, Germany) or fixed in 4% paraformaldehyde, washed two times with phosphate-buffered saline for 5 min, transferred into 25% sucrose and sectioned at 10–12 μm on a cryostat (Microm, Heidelberg, Germany). While a fragment of chicken Nx-1α was already present on databases [10], no sequences for chicken neuroligins were available. Therefore, PCR fragments homologous to the rodent NL-1 were generated with the help of partially degenerated oligonucleotide primers (sense 5′CCC CCA GAT CCT GAT GGA RCA RGG3′; anti-

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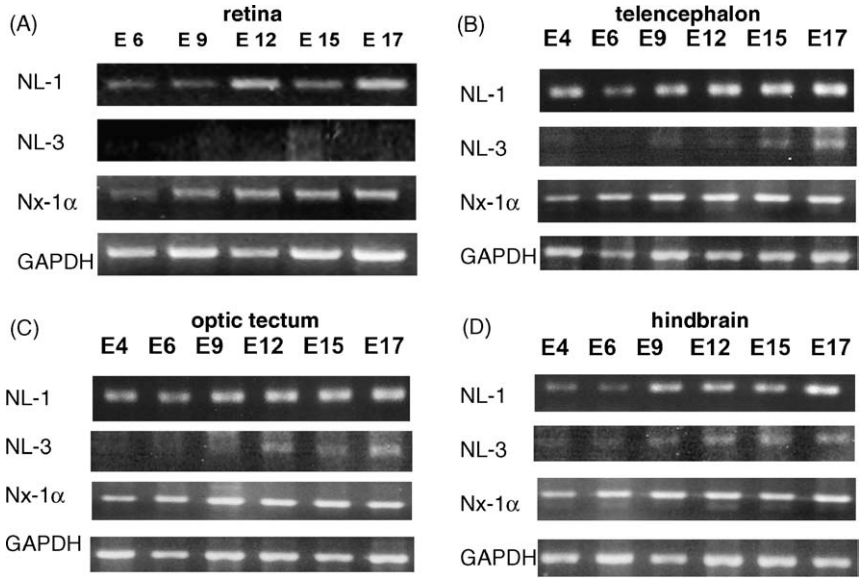

Fig. 1. Neurexin and neuroligin transcripts are detected long before synaptogenesis. Total cellular RNA was extracted from retina (A), telencephalon (B), optic tectum (C), and hindbrain (D), and subjected to RT-PCR amplification with primers selective for neurexin-1, neuroligin-1 and -3. Note continuous increase of Nx-1 in all brain regions and retina. Note also intermittent maxima of NL-1 in retina (A), while expression of NL-3 is absent in retina and could not be detected at early stages in telencephalon, optic tectum, and hindbrain (B–D). The levels of PCR products for GAPDH remain unchanged with the exception of E6 in the telencephalon. NL-1 is 369 bp, NL-3 is 272 bp, Nx-1α is 450 bp and GAPDH is 800 bp.

sense 5′TGT CGG TGG AGG GCA CYT TNG TNG T3′) or rat NL-1 specific primers (sense 5′CTG GTA CCT CAT CTG CA3′; antisense 5′CTG GTA CCT CAT CTG CA3′) and a cDNA made from RNA prepared from brain of 17- and 19-day-old chicken embryos. The PCR products were separated by agarose gel electrophoresis, purified and cloned into pCRII vector using the TA-cloning kit (Invitrogen, Karlsruhe, Germany), and sequenced. A fragment showing 86% sequence homology to the mouse NL-3 was also cloned and therefore the NL-3 expression was included in the study. Distinct regions of the mRNAs of chicken Nx-1, NL-1 and NL-3 were amplified from different embryonic brain regions and retina cDNA by PCR, using the specific primers as listed in Table 1. Cycle parameters included denaturation for 1 min at 95 °C, followed by primer annealing at 55 °C for 1 min and extension at 72 °C for 1 min, for a total of 29 cycles. In each experiment, the last cycle was followed by a 10 min elongation step at 72 °C. The experiments were done in triplicates and the results presented here are representative.

Table 1
List of primers used for the RT-PCR expression studies

Amplified gene and product size	Oligonucleotide sequence
Neuroligin-3 (272 bp)	Sense 5′CTACGGCTCCCCAACCTACT3′ Antisense 5′GCCTTGGTGTGGATGAACTT3′
Neuroligin-1 (369 bp)	Sense 5′GGTGACCCAAATCAACCAGT3′ Antisense 5′TAGTCCCTTTGATCCACTGA3′
Neurexin-1 (450 bp)	Sense 5′GGGCGTCAACTCACAATCTT3′ Antisense5′GCCAGGATACTCCCTTCCTC3′
GAPDH (800 bp)	Sense 5′CCTCTCTGGCAAAGTCCAAG3′ Antisense5′TGGCTGTCACCATTGAAGTC3′

The primers were synthesized by Carl–Roth (Germany). GAPDH, glyceraldehyde-3-phosphate dehydrogenase, is a housekeeping gene that should be equally expressed in all cells and was used as control for the cDNA synthesis; bp, base pairs.

The spatial expression pattern of Nx-1 was analyzed by in situ hybridization (ISH) on chick embryonic retina sections, corresponding to the E4 up to E19. A digoxigenin-labeled in situ hybridization riboprobe (DIG-RNA labeling kit, Roche, Germany) was generated from Nx-1 alpha chicken in pBluescript [10]. Embryonic chick retina sections, 10–12 μm thick, corresponding to the E4 up to E19 were hybridized with the above-mentioned fragment overnight at 65 °C. As control for the specificity of binding, a fragment synthesized in the 'sense'-orientation was used. No signals were detectable on sections incubated with this fragment. The staining was viewed and documented on an Axiophot microscope (Zeiss, Germany) microscope, equipped with an Intas camera. Pictures were taken using the Diskus software (Diskus 1280, CH Hilgers, Königswinter) and processed using Adobe Photoshop 7.0.

Fig. 1 shows the results of the RT-PCR expression studies. Both brain and retina were used to study the expression of Nx-1, NL-1 and NL-3. Different brain regions develop following different time-courses; therefore, RT-PCR studies for specific brain regions were done. Three different regions were chosen for this study: telencephalon, tectum and hindbrain (see Fig. 1B, C and D). In different brain regions Nx-1 and NL-1 transcripts were detected starting with E4, NL-3 appeared later, the earliest at E9 in the hindbrain. While Nx-1 showed a continuously increasing expression with increasing age, the two NLs showed a more regulated expression pattern, which for NL-1 in the retina was down- and then up-regulated (Fig. 1A). NL-3 expression showed a delayed expression in all investigated regions when compared with NL-1, and then increased to a maximum before hatching. The expression NL-1 in brain continuously increased during development. However, more fold increase was observed at the