Int. J. Dev. Biol. 46: 649-652 (2002)

Expression of neurexin ligands, the neuroligins and the neurexophilins, in the developing and adult rodent olfactory bulb

HEIDI J. CLARRIS[1], SONJA MCKEOWN[1] and BRIAN KEY*[,1,2]

[1]*Neurodevelopment Laboratory, Department of Anatomy and Developmental Biology, University of Queensland, Brisbane and* [2]*Centre for Functional and Applied Genomics, University of Queensland, Brisbane, Australia*

ABSTRACT The neurexins are a large family of neuronal cell-surface proteins believed to be involved in intercellular signalling and the formation of intercellular junctions. To begin to assess the role of these proteins in the olfactory bulb, we describe here the expression patterns of their transmembrane and secreted ligands, the neuroligins and neurexophilins, during both embryonic and postnatal development. *In situ* hybridisation showed that neuroligin 1 and 2 were expressed by second order mitral cells during early postnatal development but not in adults. The secreted ligand for α-neurexin, neurexophilin 1, was also expressed in the postnatal olfactory bulb. Neurexophilin 1 was detected in only periglomerular cells during the early postnatal period of glomerular formation but later was also expressed in mitral cells. These results suggest that neurexin-ligand interactions may be important for development and/or maturation of synaptic connections in the primary olfactory pathway.

KEY WORDS: *development, axon guidance, synaptogenesis, glomeruli*

Olfactory sensory neurons in the olfactory neuroepithelium project axons to the olfactory bulb where they terminate in specialised regions of neuropil called glomeruli. Subpopulations of olfactory sensory neurons expressing the same odorant receptor protein are dispersed randomly within one of four semi-annular zones in the nasal cavity. Despite this mosaic distribution their axons converge to approximately two topographically fixed glomeruli in the olfactory bulb (Royal and Key, 1999). While odorant receptors are involved in guiding axons to target glomeruli (Wang *et al.*, 1999; Mombaerts *et al.*, 1996), the molecules which mediate subsequent post-synaptic partner recognition and initiation of synaptogenesis remain unknown.

Molecules such as the β-neurexins and their ligands, the neuroligins, have been implicated in synaptogenesis (Song *et al.*, 1999; Scheiffele *et al.*, 2000). The neurexins are neuronal cell-surface proteins with a domain structure similar to that of cell adhesion molecules (for review, see Missler *et al.*, 1998). At least three neurexin genes, designated 1-3, are expressed in mammals and each of these has two promoters (Ushkaryov *et al.*, 1994). The upstream promoters generate long transcripts encoding α-neurexins while the downstream promoters generate shorter transcripts encoding β-neurexins (Ushkaryov *et al.*, 1994). The α- and β-neurexins differ in the size of their extracellular domains but have identical transmembrane regions and cytoplasmic tails (Ushkaryov *et al.*, 1992, 1994). The six primary neurexin transcripts undergo extensive alternative splicing, resulting in the variable addition of extra sequences at five and two sites in α- and β-neurexins respectively (Ullrich *et al.*, 1995). These sites of alternative splicing are used independently of each other, resulting in the production of potentially thousands of different neurexin isoforms (Ullrich *et al.*, 1995). It is believed that subtypes of neurons express a unique combination of neurexins (Ullrich *et al.*, 1995) and that this molecular diversity is responsible for generating specificity in synaptic connectivity.

Presently there are two known families of neurexin ligands. The neuroligins are brain-specific cell adhesion molecules and bind the β-neurexins (Ichtchenko *et al.*, 1995, 1996). Three neuroligin genes are expressed in mammals (neuroligin 1-3), and each can be alternatively spliced at the same position, but with different insert patterns (Ichtchenko *et al.*, 1996). The neurexophilins have a structure resembling neuropeptides (Eipper and Mains, 1998) and bind to α-neurexins (Petrenko *et al.*, 1996). There are at least four genes encoding neurexophilins (Petrenko *et al.*, 1996; Missler *et al.*, 1998). Presently, there is no direct evidence for a signalling function, but it is possible that neurexophilins serve as neuropeptides while α-neurexins act as their receptors.

In preliminary experiments we used reverse transcriptase polymerase chain reaction (RT-PCR) to examine the expres-

Abbreviations used in this paper: PCR, polymerase chain reaction; RT-PCR, reverse transcriptase PCR.

***Address correspondence to:** Dr. Brian Key. Neurodevelopment Laboratory, Department of Anatomy and Developmental Biology, University of Queensland, Brisbane 4072, Australia: Tel: +7-3365-2955. Fax: +7-3365-2955. e-mail: brian.key@uq.edu.au

0214-6282/2002/$25.00

Printed in Spain
www.ijdb.ehu.es

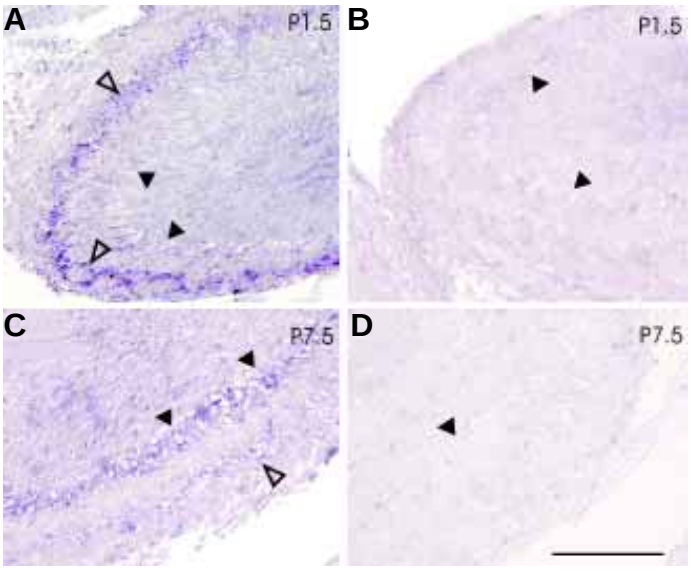


Fig. 1. Neurexophilin 1 expression in the developing mouse olfactory bulb. *Coronal sections through the P1.5* **(A,B)** *and P7.5* **(C,D)** *mouse olfactory bulb. Sections were hybridised with neurexophilin 1 antisense (A,C) or sense (B,D) RNA probes. Signal for neurexophilin 1 is present in periglomerular cells of the P1.5 mouse (A,B), and in both periglomerular and mitral cells of the P7.5 mouse (C,D). Closed arrowheads point to the mitral cell layer.*

sion of neurexins in the mouse olfactory mucosa using total RNA isolated from E16.5, P3.5, P7.5 and adult mice. PCR primers were designed to distinguish between neurexins 1α, 1β, and 2α (Table 1). Following PCR, amplification products were gel eluted, subcloned into Bluescript and sequenced to confirm their identity as neurexin isoforms. This approach revealed the expression of *neurexin 1α, 1β* and *2α* mRNA in olfactory mucosa at all ages examined (not shown). Negative control reactions lacking reverse transcriptase confirmed the absence of non-specific reaction products. Neurexins are most likely expressed by the olfactory sensory neurons since these molecules are only expressed by neurons (reviewed in Missler *et al.*, 1998). This conclusion is also consistent with previous reports localising neurexins to glutamatergic neurons (Ichtchenko *et al.*, 1998) since, at least in mouse, olfactory sensory neurons release glutamate as their neurotransmitter (Griff *et al.*, 2000).

Next we examined the *in situ* expression of the α-neurexin ligand, neuroexophilin 1 within the mouse olfactory bulb. Neuroexophilin 1 was not detected in the olfactory bulb prior to birth (not shown). *In situ* hybridization revealed *neurexophilin 1* was first expressed by periglomerular cells at P1.5 (Fig. 1 A,B). Later at P7.5, expression was also detected in the mitral cell layer (Fig. 1 C,D). This expression continues into adulthood (Petrenko *et al.*, 1996). Since *neuroligins 1* and *2* have previously been cloned in rat we next analysed the expression of these β-neurexin ligands in the rat olfactory bulb. At E18.5, *neuroligins 1* and *2* were not detected above background levels (Fig. 2 A,B; Fig. 3 A,B). However, during the next week of development both ligands began to be expressed by mitral cells (Fig. 2 C-F; Fig. 3 C,D). Neuroligin 1 and 2 were not detected in the adult olfactory bulb (Fig. 2 G,H; Fig. 3 E,F).

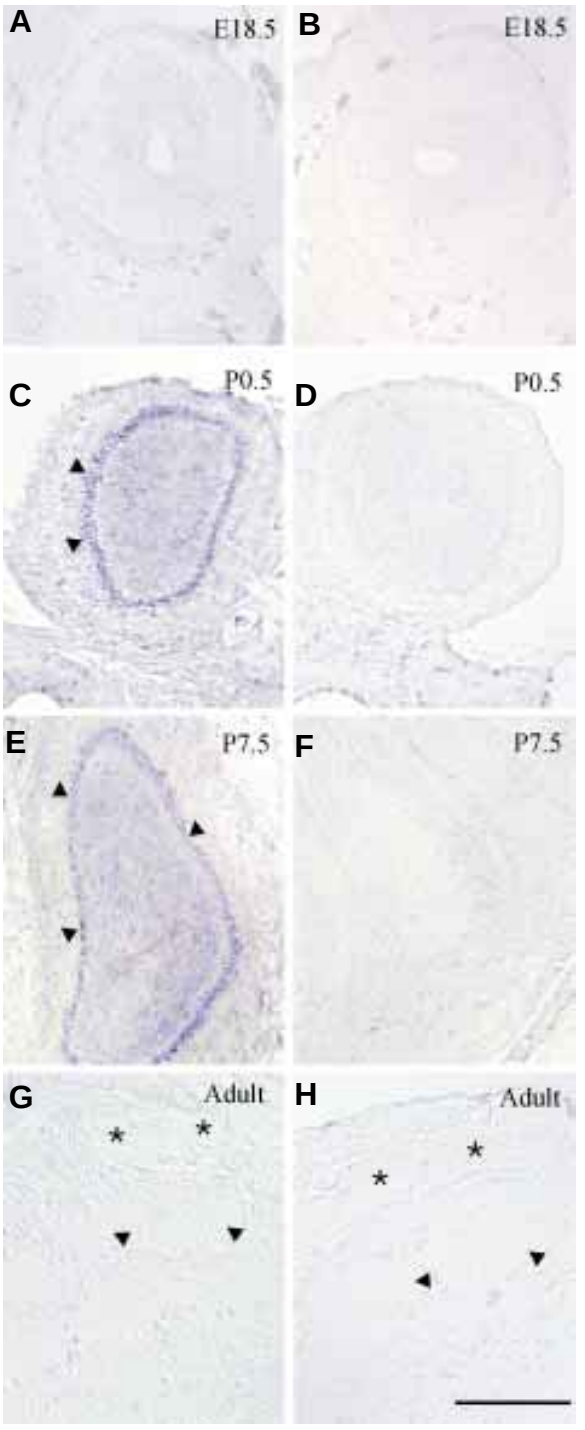
During postnatal development we found the transmembrane β-neurexin ligands, *neuroligin 1* and *2* were expressed by second order mitral cells, whose primary dendrites make synaptic contact with primary olfactory axons within glomeruli. It has been proposed that at the level of the synapse, β-neurexin present in the presynaptic axonal membrane binds to transmembrane neuroligin ligands in target neurons (Butz *et al.*, 1998). A series of protein-protein interactions then leads to the recruitment of synaptic vesicles and the formation of synaptic specialisations (Scheiffele *et al.*, 2000). It is possible that β-neurexin-neuroligin interactions play an important role in the formation and/or maintenance of olfactory synapses. Interestingly, neuroligin 3 has been previously reported to be selectively expressed by ensheathing cells in the olfactory nerve pathway (Gilbert *et al.*, 2001). This neuroligin may be interacting with neurexins on olfactory axons to promote glia cell ensheathment of axon bundles. In contrast to the neuroligins, the secreted α-neurexin ligand, neurexophilin 1, was detected within periglomerular cells in the olfactory bulb. This was consistent with previous reports of neurexophilin expression in inhibitory GABAergic interneurons (Petrenko *et al.*, 1996). The expression of neuroligins and neurexophilins during the early postnatal period and not in embryonic olfactory bulb suggests that these molecules are more likely involved in late glomerular formation and maturation. Glomeruli become morphologically defined at birth and become adult-like during the second postnatal week (Key, 1998; Royal and Key, 1999).

The initial data presented in this study suggest that these molecules have specific spatiotemporal roles in the development of the olfactory nerve projection. To our knowledge, we have for the first time demonstrated the expression of neurexins and their ligands in a contiguous neuronal pathway. Interestingly, both _- and _-neurexins have recently been shown to be receptors for dystroglycan, a glycoprotein involved in linking the extracellular matrix to the cytoskeleton (Sugita *et al.*, 2001). The expression of dystroglycan and other members of the dystrophin associated protein complex by mitral cells (Gorecki *et al.*, 1994; Zaccaria *et al.*, 2001) raises the intriguing possibility that neurexin-dystroglycan

TABLE 1

SEQUENCE OF OLIGONUCLEOTIDE PRIMERS USED FOR GENERATING PCR PRODUCTS

Target Gene	Name	Primer sequence	Positions	Fragment length (bp)
Neurexin 1α	NX1α Forward	GAGGATCCGAA/GTGGTGT/CCAC/TGTGGATTGAGGATCCGAA/GTGG	2578-2596	502
	NX1α Reverse	TGT/CCAC/TGTGGATTGAGAATTCTCC/TTCTGCC/TTCCGTG/ATGCAT	3080-3061	
Neurexin 1β	NX1β Forward	GAGGATCCCGCCATGTACCAGAGGATGGGGAATTCGATATACGTC	120-139	268
	NX1β Reverse	GTCCCAGCGGAGGATCCCGCCATGTACCAGAGGATGGGGAATTCGATATACGTCGTCCCAGCG	387-368	
Neurexin 2α	NX2α Forward	GAGGATCCGAA/GTGGTGT/CCAC/TGTGGATTGAGAATTCCCCAAGAC	1975-1994	505
	NX2α Reverse	GCCCAGCAAGGC	2480-2461	
Neuroligin 1	NL1 Forward	TGCTCTAGACAATCTGCTGACTTTATCCCGCGGGATCCAAACTTCAAC	1702-1721	384
	NL1 Reverse	CCTTCCCC	2086-2069	
Neuroligin 2	NL2 Forward	TGCTCTAGACTGATCCTCATGCAACAGGCGGGATCCGGCAGTGGTGG	1642-1660	370
	NL2 Reverse	TAAAAAG	2012-1995	
Neurexophilin 1	Neurexo1 Fwd.	CGGGGTACCAATTCTAAGCCTCTCAGCAAGTGCTCTAGAAAATCACCC	57-77	414
	Neurexo1 Rever.	CATCCAAAC	470-453	



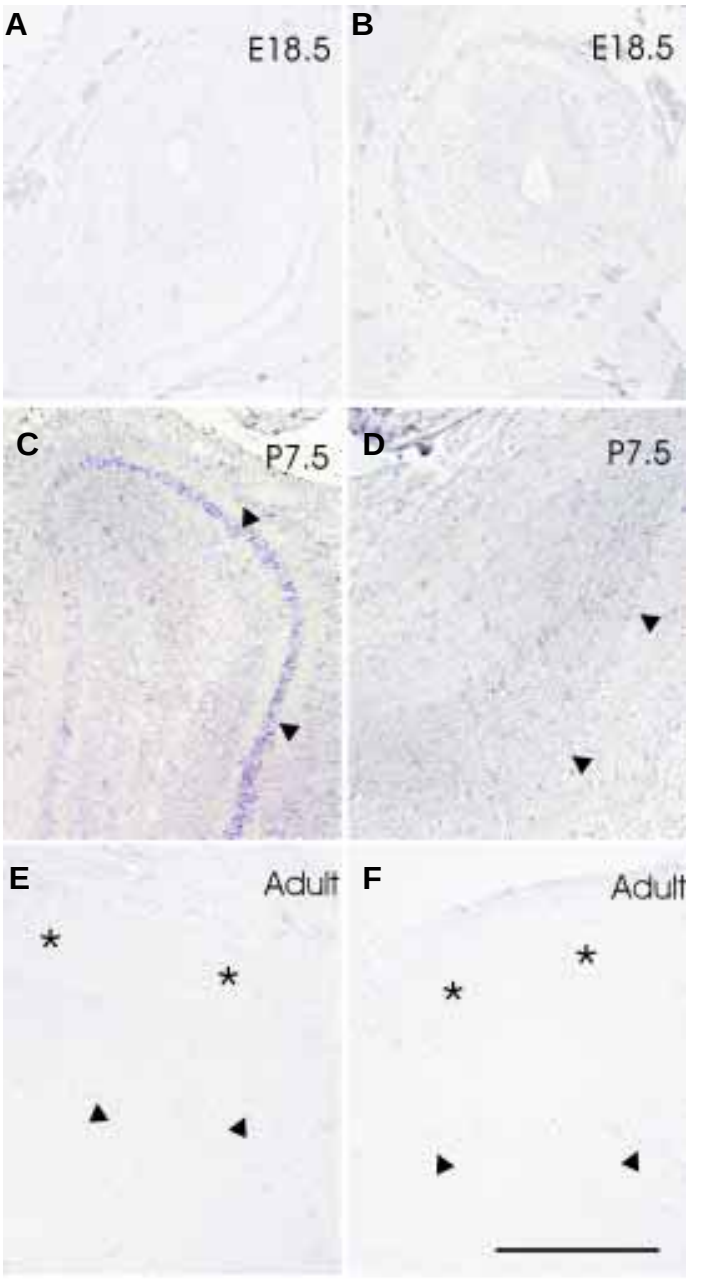


Fig. 2. (Left) Neuroligin 1 expression in the developing rat olfactory bulb. *Coronal sections through the E18.5* **(A, B)**, *P0.5* **(C, D)**, *P7.5* **(E, F)** *and adult* **(G, H)** *rat olfactory bulb. Sections were hybridised with neuroligin 1 antisense (A,C,E,G) or sense (B,D,F,H) RNA probes. Expression is absent from bulbs at E18.5 and adult. Signal for neuroligin 1 is observed within the mitral cell layer of P0.5 and P7.5 olfactory bulbs. Closed arrowheads point to the mitral cell layer. Stars mark glomeruli. Scale bar represents 250 μm (A,B), 300 μm (C-F) and 100 μm (G,H).*

Fig. 3. (Right) Neuroligin 2 expression in the developing rat olfactory bulb. *Coronal sections through the E18.5* **(A,B)**, *P7.5* **(C,D)** *and adult* **(E,F)** *rat olfactory bulb. Sections were hybridised with neuroligin 2 antisense (A,C,E) or sense (B,D,F) RNA probes. Expression is absent from bulbs at E18.5 and adult. Signal for neuroligin 2 is observed within the mitral cell layer of P7.5 bulbs. Closed arrowheads point to the mitral cell layer. Stars mark glomeruli. Scale bar represents 250 μm (A-D) and 100 μm (E,F).*

interactions may also be mediating cell adhesion at the level of the olfactory glomerulus, independent of either neuroligins or neurexophilins. Further analysis of the precise role of the neurexins in glomerular formation will require examination of protein expression at the level of the synapse. The production of neurexin gain-of-function and loss-of-function transgenic mice will also be necessary to understand the importance of these molecules in this region of the nervous system.

Experimental Procedures

Experimental Animals

Timed pregnant Sprague Dawley rats were sacrificed by cervical dislocation. Embryonic (E) rats were removed by caesarean section at E19.5. Postnatal (P) rats were collected at P0.5, P7.5, P14.5 and as adults. The morning following conception was designated as E0.5. Timed pregnant C57BL/6 mice were sacrificed by cervical dislocation. Embryos were collected at E16.5. Postnatal mice were collected at P0.5, P3.5, P7.5 and as adults. Early embryonic mice (E16.5) were fixed by immersion in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), pH7.4, for 24 h. The heads of all older mice were immersed in fixative for 24 h and decalcified in 20% EDTA solution.

Reverse Transcriptase Polymerase Chain Reaction

To examine the expression of neurexin, neuroligin and neurexophilin mRNA, distinct regions of the mRNAs were amplified from cDNA by the polymerase chain reaction using specific oligonucleotide primers (Table 1). Total RNA was isolated from mouse and rat olfactory mucosa, bulb and brain using an RNA isolation kit (QIAGEN, CA). Single-stranded cDNA was synthesised on 2-10 μg of total RNA in a standard reverse transcriptase (RT) reaction primed by oligo(dT) (GIBCO Bethesda Research Laboratories, BRL, Paisley, UK). A typical PCR mixture contained in 25 μl; 2 μl cDNA template, 1 X PCR buffer (GIBCO BRL), 2 mM $MgCl_2$, 100 pmol each primer, and 2.5U Platinum Taq DNA polymerase (GIBCO BRL). PCR fragments were generated by 30 cycles of denaturation (1 min, 94 ^oC), annealing (1.0 min, 60 ^oC) and extension (1.0 min, 72 ^oC). Since the complete sequences of mouse neurexins and neurexophilins were available (EMBL GeneBank), we performed RT-PCR using oligonucleotide sequences designed against mouse. To amplify neurexin isoforms, we used primer sequences previously designed by Puschel and Betz (1995). The complete sequences for neuroligin 1 and 2 cDNAs were only available in rat (EMBL GeneBank). Therefore, neuroligin 1 and 2 isoforms were amplified from RNA derived from rat tissue. All oligonucleotide primer sequences included 5' restriction enzyme recognition sequences for subsequent subcloning and sequencing of PCR products. Primer sequences and corresponding positions within neurexin, neuroligin and neurexophilin cDNA sequences are shown in Table 1.

Generation of RNA Probes and In Situ *Hybridization*

Neuroligin and *neurexophilin* RT-PCR fragments generated from P3.5 rat and mouse olfactory bulb respectively were digested with XbaI and BamHI (Promega Corporation, WI), purified, and subcloned into Bluescript (Stratagene Cloning Systems, CA). The identity of the plasmids was confirmed by partial DNA sequencing (Pharmacia T7 sequencing kit). Digoxigenin (DIG)-labeled antisense and sense RNA probes were transcribed from 1 μg plasmid DNA using T3 and T7 RNA polymerases (Promega Corporation), according to the manufacturer's instructions. All *in situ* hybridisation reactions were performed as previously described (Puche and Key, 1995).

Acknowledgements

This work was supported by an Australian National Health and Medical Research Council grant to BK. HJC was supported by a National Health and Medical Research Council Peter Doherty Fellowship.

References

BUTZ, S., OKAMOTO, M. and SUDHOF, T.C. (1998). A tripartite protein complex with the potential to couple synaptic vesicle exocytosis to cell adhesion in brain. *Cell* 94: 773-82.

EIPPER, B.A. and MAINS, R.E. (1980). Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocrin. Rev.* 1: 1-27.

GILBERT, M., SMITH, J., ROSKAMS, A.J. and AULD, V.J. (2001). Neuroligin 3 is a vertebrate gliotactin expressed in the olfactory ensheathing glia, a growth-promoting class of macroglia. *Glia* 34: 151-164.

GRIFF, E.R., GREER, C.A., MARGOLIS, F., ENNIS, M. and SHIPLEY, M.T. (2000). Ultrastructural characteristics and conduction velocity of olfactory receptor neuron axons in the olfactory marker protein-null mouse. *Brain Res.* 866: 227-36.

GORECKI, D.C., DERRY, J.M. and BARNARD, E.A. (1994). Dystroglycan: brain localization and chromosome mapping in the mouse. *Human Mol. Gen.* 3: 1589-97.

ICHTCHENKO, K., HATA, Y., NGUYEN, T., ULLRICH, B., MISSLER, M., MOOMAW, C. and SUDHOF, T.C. (1995). Neuroligin 1: a splice site-specific ligand for beta-neurexins. *Cell* 81: 435-43.

ICHTCHENKO, K., KHVOTCHEV, M., KIYATKIN, N., SIMPSON, L., SUGITA, S. and SUDHOF, T.C. (1998). alpha-latrotoxin action probed with recombinant toxin: receptors recruit alpha-latrotoxin but do not transduce an exocytotic signal. *EMBO J.* 17: 6188-6199.

ICHTCHENKO, K., NGUYEN, T. and SUDHOF, T.C. (1996). Structures, alternative splicing, and neurexin binding of multiple neuroligins. *J. Biol. Chem.* 271: 2676-82.

KEY, B. (1998). Molecular development of the olfactory nerve pathway. *Ann. N.Y. Acad. Sci.* 855: 76-82.

MISSLER, M., FERNANDEZ-CHACON, R. and SUDHOF, T.C. (1998). The making of neurexins. *J. Neurochem.* 71: 1339-1347.

MOMBAERTS, P., WANG, F., DULAC, C., CHAO, S.K., NEMES, A., MENDELSOHN, M., EDMONDSON, J. and AXEL, R. (1996). Visualizing an olfactory sensory map. *Cell* 87: 675-686.

PETRENKO, A.G., ULLRICH, B., MISSLER, M., KRASNOPEROV, V., ROSAHL, T.W. and SUDHOF, T.C. (1996). Structure and evolution of neurexophilin. *J. Neurosci.* 16: 4360-4369.

PUCHE, A. and KEY, B. (1995). Identification of cells expressing galectin-1, a galactose binding receptor, in the rat olfactory system. *J. Comp. Neurol.* 357: 513-523.

ROYAL, S.J. and KEY, B. (1999). Development of P2 olfactory glomeruli in P2-internal ribosome entry site-tau-LacZ transgenic mice. *J. Neurosci.* 19: 9856-9864.

SCHEIFFELE, P., FAN, J., CHOIH, J., FETTER, R. and SERAFINI, T. (2000) Neuroligin expressed in nonneuronal cells triggers presynaptic development in contacting axons. *Cell* 101: 657-669.

SONG, J.Y., ICHTCHENKO, K., SUDHOF, T.C. and BROSE, N. (1999). Neuroligin 1 is a postsynaptic cell-adhesion molecule of excitatory synapses. *Proc. Nat. Acad. Sci. USA* 96: 1100-5.

SUGITA, S., SAITO, F., TANG, J., SATZ, J., CAMPBELL, K. and SUDHOF, T.C. (2001). A stoichiometric complex of neurexins and dystroglycan in brain. *J. Cell Biol.* 154: 435-45.

ULLRICH, B., USHKARYOV, Y.A. and SUDHOF, T.C. (1995). Cartography of neurexins: more than 1000 isoforms generated by alternative splicing and expressed in distinct subsets of neurons. *Neuron* 14: 497-507.

USHKARYOV, Y.A., HATA, Y., ICHTCHENKO, K., MOOMAW, C., AFENDIS, S., SLAUGHTER, C.A. and SUDHOF, T.C. (1994) Conserved domain structure of beta-neurexins. Unusual cleaved signal sequences in receptor-like neuronal cell-surface proteins. *J. Biol. Chem.* 269: 1987-1992.

USHKARYOV, Y.A., PETRENKO, A.G., GEPPERT, M. and SUDHOF, T.C. (1992). Neurexins: synaptic cell surface proteins related to the alpha-latrotoxin receptor and laminin. *Science* 257: 50-56.

WANG, F., NEMES, A., MENDELSOHN, M. and AXEL, R. (1998) Odorant receptors govern the formation of a topographic map. *Cell* 93: 47-60.

ZACCARIA, M.L., DI TOMMASO, F., BRANCACCIO, A., PAGGI, P. and PETRUCCI, T.C. (2001). Dystroglycan distribution in adult mouse brain: A light and electron microscopy study. *Neurosci.* 104: 311-324.