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Sequential onset of pre-synaptic molecules during olfactory sensory neurons maturation

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Abstract

Differentiated olfactory sensory neurons express specific pre-synaptic proteins, including enzymes involved in neurotransmitter transport and proteins involved in the trafficking and release of synaptic vesicles. Studying the regulation of these pre-synaptic proteins will help to elucidate the pre-synaptic differentiation process that ultimately leads to synapse formation. It has been postulated that the formation of a synapse between the axons of the sensory neurons and the dendrites of second order neurons in the olfactory bulb is a critical step in the processes of sensory neuron maturation. One approach to study the relationship between synaptogenesis and sensory neuron maturation is to examine the expression patterns of synaptic molecules through the olfactory neuron lineage. To this end we designed specific *in situ* hybridization probes to target messengers for proteins involved in pre-synaptic vesicle release.

Our findings show that, as they mature, mouse olfactory neurons sequentially express specific pre-synaptic genes. Furthermore, the different patterns of expression of these pre-synaptic genes suggest the existence of discrete steps in pre-synaptic development: genes encoding proteins involved in scaffolding show an early onset of expression whereas expression of genes encoding proteins involved in the regulation of vesicle release starts later. In particular, the signature molecule for glutamatergic neurons Vesicle Glutamate Transporter 2 shows the latest onset of expression. In addition, contact with the targets in the olfactory bulb is not controlling pre-synaptic protein gene expression, suggesting that olfactory sensory neurons follow an intrinsic program of development.

Keywords

olfactory epithelium; olfactory sensory neuron maturation; bulbectomy; synapse formation; Vesicle Glutamate Transporter 2

INTRODUCTION

Synapse formation involves the recognition of target cells by pre-synaptic neurons followed by the concerted differentiation of pre- and post-synaptic partners to form mature synapses. From a molecular perspective, this process involves the recruitment of numerous molecules

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to sites of physical contact between axons and dendrites and their precisely timed assimilation into functional assemblies leading to the final structure of the synapse. In particular, excitatory pre-synaptic differentiation includes the clustering of synaptic vesicles (SVs) and the formation of active zones, specialized regions within the pre-synaptic plasma membrane where SVs dock, fuse and release neurotransmitter. The active zone is tightly associated with an electron-dense cytoskeletal matrix referred as the cytomatrix of the active zone (CAZ). Not surprisingly, many types of molecules are highly concentrated at glutamatergic pre-synaptic terminals: active zone components (including the CAZ components bassoon and piccolo), proteins involved in neurotransmitter transport, molecules involved in the trafficking and release of SVs, and trans-synaptic adhesion molecules (for review on synaptogenesis see McAllister, 2007; Ziv, 2001). The study of the regulation of these pre-synaptic proteins should help to elucidate the still poorly understood process of pre-synaptic differentiation.

The mammalian olfactory epithelium (OE) possesses the rare capacity of continuous neurogenesis during adulthood: precursor cells continuously divide and differentiate into mature olfactory sensory neurons (OSNs) under physiological conditions and throughout the organism's life. Newborn sensory neurons project their axons into the olfactory bulb (OB) to establish functional synapses with tufted, mitral and periglomerular cells within specialized structures known as glomeruli. Ultrastructural aspects of synapse formation in the glomerular layer have been characterized during development (Blanchart et al., 2008; Hinds and Hinds, 1976). Electron microscopy studies have shown that in mice OSN axons first enter the bulb at embryonic day 11 (E11), the first synapses are observed at E13 and OMP immunoreactivity (a mature cellular marker for olfactory sensory neurons, see below) is first observed at E14 (Farbman and Margolis, 1980). However the intrinsic and/or extrinsic signals leading to sensory neuron maturation, and how this later relates to pre-synaptic differentiation, remain to be explored.

Identifying synapse-associated molecules expressed by sensory neurons and changes in their expression patterns along the OE neuronal lineage is a first step toward understanding the relation between synapse formation and sensory neuron maturation. A proposed model for the neuronal lineage in the olfactory epithelium includes MASH1-expressing progenitors located basally in the neuroepithelium that give rise, after rounds of division, to receptor neurons. Within this population of postmitotic neurons, mature olfactory sensory neurons, expressing OMP, are located above immature olfactory sensory neurons, expressing growth associated protein 43 (GAP43) (Calof et al., 2002). The pseudo-stratified organization of the olfactory epithelium makes it an ideal model to study the differentiation and maturation of neurons, since the entire lineage of OSNs, from progenitors to immature to mature cells, can be readily seen in a single OE tissue section.

By using a panel of specific *in situ* hybridization probes, we assessed the expression patterns of mRNA for proteins involved in the pre-synaptic vesicle release machinery in the mouse olfactory epithelium. Our results indicated a sequential onset of expression for the pre-synaptic molecules as sensory neurons mature. Genes encoding for proteins that play a structural role at the active zone showed an early onset of expression, whereas genes encoding for proteins associated with synaptic vesicles showed a later onset of expression.

In particular, a signature molecule for glutamatergic neurons, the Vesicle Glutamate Transporter 2 (VGluT2) was expressed by fully mature olfactory sensory neurons only, showing the latest onset of expression. Also, the expression of all pre-synaptic molecules so far tested was restored after recovery from surgical ablation of the olfactory bulb, suggesting that olfactory sensory neurons would mature as pre-synaptic cells independently of their target. Our data set the stage for understanding the molecular events underlying the differentiation and pre-synaptic maturation of olfactory sensory neurons.

MATERIALS AND METHODS

Animals

SVE129 wild-type animals were obtained from Taconic, Farms, Inc. (Germantown, NY). All animals were housed at Columbia University in accordance with institutional requirements for animal care.

Surgical ablation of the olfactory bulb

Unilateral bulbectomies were performed on 8-week old male mice. Mice were anaesthetized by injection with Ketamine/Xylazine (18 mg/ml and 2 mg/ml, respectively) and a midline incision was made to expose the cranial bone. A small opening just above the right olfactory bulb was made with a drill, and using gentle suction the right olfactory bulb was removed. The cavity vacated by the bulb was then packed with sterile Gel Foam (UpJohn, Kalamazoo, MI). Finally, the incision in the skin over the snout was closed with Vetbond (3M Animal Care Products, St. Paul, MN). Animals were allowed to recover for 30 days after surgery. The degree of the lesion was verified visually and microscopically. We only used animals where the degree of lesion would be complete.

In situ Hybridization

In situ hybridizations were performed on 5-day, 20-day and ~3 month old mouse olfactory epithelium (OE) tissue. In brief, mice were transcardially perfused with 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH 8.00. Olfactory epithelial tissue was dissected out and postfixed overnight. The epithelial tissue was then decalcified for two days in 0.5 M EDTA pH 8.00 treated with 0.1% of diethyl pyrocarbonate (DEPC) if the animals were 20 days old and for four days if the animals were older, followed by an overnight immersion in 30% sucrose-PBS as a preparation for cryoprotection, all at 4°C. Then 10 µm thick coronal cryosections were cut and warmed to 55°C. Slides were fixed in 4% PFA, washed in DEPC-PBS, treated with 0.3% H₂O₂ in PBS for 30 minutes at room temperature in order to block endogenous peroxidases if the slides were processed for two color *in situ* hybridization, treated with 10 µg/ml proteinase K at 37°C, fixed once more in 4% PFA, washed with PBS, incubated in 0.1 M triethanolamine with 0.03% acetic anhydride and washed again. Slides were dehydrated in 60%, 80%, 95%, and twice in 100% ethanol and then each slide was hybridized overnight at 63°C with 0.5–1 µl of biotin and/or 0.5–1 µl of digoxigenin (DIG)-labeled probes in 200 µl of hybridization solution (50% formamide, 10 mM Tris-Cl pH 8.0, 200 µg/ml yeast tRNA, 10% dextran sulfate, 1X Denhardt's solution, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1 mM EDTA pH 8.0). After hybridization, slides were washed in 2X SSC, 50% formamide, treated with 20 µg/ml

RNase A at 37°C to remove any un-hybridized probe and finally washed in a series of 2X, 0.2X and 0.1X SSC. The slides were blocked in 0.5% NEN buffer (Perkin-Elmer, Boston, MA) for 1 hour and then incubated for 48 hours at 4°C in alkaline phosphatase-conjugated anti-DIG diluted 1:200 in 0.5% NEN buffer (Roche, Indianapolis, IN). Probes targeting olfactory neurons cellular markers were labeled with biotin and probes targeting pre-synaptic molecules were labeled with DIG. For two color fluorescent in situ hybridization biotin and DIG labeled probes were detected sequentially as described in Ishii et al (Ishii et al., 2004). First, biotin labeled probes were detected using the TSA biotin system (Perkin Elmer, Boston, MA) followed by an incubation with the Streptavidin Alexa Fluor 488 conjugate (Molecular probes, Eugene, OR) diluted 1:300 in the blocking buffer provided by the manufacturer. Second, DIG labeled probes were detected using the HNPP fluorescent detection set (Roche, Mannheim, Germany) incubating with the HNPP/Fast Red solution for 3×30 minutes due to the low transcript level. Although we quenched endogenous peroxidases as suggested by the manufacturer, we observed high background in the probes detected with the TSA biotin system. This non-specific label is localized above the most apical cell layer of the olfactory epithelium, in the mucus and cilia, and in the lamina propria. For chromogenic *in situ* hybridization slides were washed in 150 mM NaCl, 100 mM Tris-Cl pH 7.5, 0.05% Tween 20 and then incubated with 66 µl NBT and 33µl BCIP (NBT/BCIP, Promega, Madison, WI) in 10 ml of 100 mM NaCl, 100 mM Tris-Cl pH 9.5, 50 mM MgCl₂ until color develops.

***In situ* probes**

Probes for bassoon, gap43, Munc18-1, omp, piccolo, SNAP25, synaptophysin, synaptotagmin 1, syntaxin 1A and VAMP2 were designed to target the mRNA regions described on Table 1 and templates were cloned from olfactory epithelium or brain cDNA into pCRII-TOPO (Invitrogen, Carlsbad, CA). Probes for synapsin 1, Vesicle Glutamate Transporter 2 (VGluT2) and MASH1 were generated from cDNA clones (IMAGE ID#s 5368098, 5357143 and 6415061, respectively) obtained from Open Biosystems (Huntsville, AL). Probes for synapsin 1 and VGluT2 were generated from cDNA clones digested with BglII and HpaI respectively (see Table 1). Probe for MASH1 was generated from full-length cDNA clone. The pan-α-neurexin probe template was generously provided by Dr. Peter Scheiffele (University of Basel, Switzerland). The sequence from pan-α-neurexin probe aligns with nucleotides 1,924–2,611 from neurexin 1 with 100% identity, nucleotides 901–1,595 from neurexin 2 with 70% identity and nucleotides 337–1,013 from neurexin 3 with 74% identity (see Table 1) (alignments were performed by using the Align Tool from NCBI <http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Probes preparation were done according to Ishii et al, 2004. In brief template DNA, RNA labeling mixture (Roche, Mannheim, Germany), 10x transcription buffer (Roche, Mannheim, Germany), RNasin (Roche, Mannheim, Germany) and RNA polymerase (Roche, Mannheim, Germany) were incubated at 37° for 2 hours. Probes were precipitated and pellets were dissolved in 25 µl DEPC treated water. Probes specificity were determined by testing sense and anti-sense probes simultaneously (see Supplementary Figures 1 and 2).

Immunohistochemistry

Mice were transcardially perfused with 4% PFA in PBS. Olfactory epithelial tissue was dissected out and postfixed for 2 hours, followed by decalcification in 0.5 M EDTA pH 8.00 performed over two days for 20 days old mice and over four days for older animals and by overnight immersion in 30% sucrose-PBS, all at 4°C. Immunohistochemistry was performed on 16 µm cryosections on slides (Superfrost, Fischer, Fair Lawn, NJ). Slides were warmed to 55°C for 10 minutes followed by steaming in 0.01 M sodium citrate pH 6.0 for 10 minutes. The slides were cooled then rinsed in PBSTx (0.1% Triton X in PBS). The sections were blocked in 10% Normal Donkey Serum (NDS) in PBSTx for 2 hours and then incubated in primary antibody mixtures in 2.5% normal donkey serum in PBSTx overnight at 4°C. Sections were washed in PBSTx (4×10 minutes) and then incubated in secondary antibody in 2.5% NDS-PBSTx at room temperature for 1–2 hours. The secondary antibodies were AlexaFluor-conjugated 488 or 594-donkey anti-rabbit or anti-goat diluted 1:750 in blocking buffer (Molecular Probes, Eugene, OR). Slides were washed 2×10 minutes in PBSTx and 2×10 minutes in PBS before counterstaining with TOTO-3 diluted 1:10,000 in PBS (Molecular probes, Eugene, OR). Mounting of the sections was performed using Vectashield (Vector, Burlingame, CA).

Primary antibodies

Goat anti-OMP (Wako, Dallas, TX, #544–10001) was used in immunohistochemistry at 1:2,000. This polyclonal serum was obtained from a goat through multiple immunizations with rodent olfactory marker protein (OMP). It stains a single band of 19 kDa in Western blot, and its specificity in immunohistochemistry has been verified by pre-adsorption of the antiserum with high-performance liquid chromatography (HPLC)-purified OMP, which abolishes all staining (Baker et al., 1989).

Rabbit anti-VGluT2 (Synaptic Systems, Goettingen, Germany, #135–403) was used at 1:1,000. This polyclonal serum was obtained from a rabbit upon immunization with a synthetic antigen containing the amino acid residues 510–582 of rat VGluT2. VGluT2 antibody recognizes a single band of 65 kDa on immunoblot. This immunoreactive band is abolished after incubation of the antiserum with the antigen used for immunization (Takamori et al., 2001).

Microscopy

Tissue sections were imaged on an Olympus confocal microscope (Olympus FluoView 600) and analyzed by using Fluoview, ImageJ, and Adobe Photoshop. Images were not modified other than to balance brightness and contrast. All of the genes tested in the present study are expressed in all four zones of mouse olfactory epithelium. Images were taken from septum of mouse OE.

RESULTS

Expression of mRNAs for pre-synaptic molecules in the Olfactory Epithelium

Mouse olfactory epithelium (OE) tissue was processed for fluorescent *in situ* hybridization. In order to establish the temporal onset of pre-synaptic differentiation during olfactory

sensory neuron lineage, we combined specific probes targeting genes encoding cellular markers expressed along the sensory neuron lineage and specific probes targeting genes encoding molecules enriched in pre-synaptic terminals. In our study we sought to examine pre-synaptic molecules with diverse functions. We therefore targeted genes that would code for active zone components (piccolo and bassoon), SNARE proteins (syntaxin 1A, VAMP2 and SNAP25), a SNARE associated protein (Munc18-1), vesicle associated proteins (synapsin 1, synaptotagmin 1 and synaptophysin), a neurotransmitter transporter protein (Vesicle Glutamate Transporter 2 or VGluT2) and trans-synaptic adhesion proteins (α -neurexins, Note: pan- α -neurexin probe recognizes the three α -neurexins gene transcripts) (Bennett et al., 1992; Cases-Langhoff et al., 1996; Chin et al., 1995; Edelman et al., 1995; Geppert et al., 1994; Lise and El-Husseini, 2006; Sollner et al., 1993; Takamori et al., 2001; tom Dieck et al., 1998; Toonen and Verhage, 2007). We observed three distinct patterns of expression of pre-synaptic molecules that depended upon when during OSN lineage the onset of expression took place.

We observed that the mRNAs for some synaptic molecules coincided with that of MASH1, a progenitor cell marker for olfactory sensory neurons (Figure 1) (Calof et al., 2002). Examples included bassoon, piccolo, Munc18-1 and syntaxin 1A. This result suggests that the onset of expression for these genes occurs very early in the olfactory sensory neuron lineage, when progenitor cells are at the earliest transit amplifying stage. Importantly, these mRNAs continued to be expressed through later stages in sensory neuron lineage. We should note that Sammeta et al., 2007 reported that piccolo was expressed by basal and immature OSNs only by using an *in situ* hybridization probe that targeted a different region of piccolo mRNA than the one targeted by our probe (Sammeta et al., 2007). In order to compare both results side by side, we tested simultaneously the probe for piccolo described in Sammeta et al, 2007 (which targets the nucleotides 13,793–14,244 from piccolo mRNA NM_011995) and the probe for piccolo used in Figure 1 (which targets the nucleotides 15,204–16,029 from piccolo mRNA NM_011995) (Supplementary Figure 2). We observed that qualitatively both probes are similar but that the positive signal obtained with our probe for piccolo was stronger, thereby explaining the apparent discrepancy. We should also note that with our piccolo *in situ* hybridization probe we observed a weak positive signal in sustentacular cells, a non-neuronal secretory cell type located in the most apical layer of the OE (see Figure 1D). Since there is evidence of piccolo expression by other types of secretory cells, such as the pancreatic beta cells where it is involved in secretion of insulin upon stimuli (Fujimoto et al., 2002), we speculated that piccolo expression by sustentacular cells has a different role than that of piccolo expression by OSNs.

Other mRNAs were not detected in MASH1+ neuronal progenitors (Figure 2) and their expression came on later in the olfactory sensory neuron lineage, when cells differentiated into GAP43-expressing immature receptor neurons and begun to project their axons outside the epithelium (Figure 3) (Verhaagen et al., 1989). Examples of genes whose expression overlapped with the immature cell marker GAP43 are α -neurexins, synapsin 1, VAMP2, SNAP25, synaptotagmin 1 and synaptophysin. The mRNA for these genes also continued to be expressed through later stages in olfactory sensory neuron lineage.

Finally, we examined the expression of the glutamate transporter VGluT2, a transporter responsible for the accumulation and storage of glutamate within synaptic vesicles and known to be restricted to glutamatergic neurons. In the olfactory epithelium, VGluT2 mRNA showed a late onset of expression. Indeed, VGluT2 mRNA was absent from immature GAP43+ cells and was restricted only to mature OMP+ cells (Figure 4, C and F) (Farbman and Margolis, 1980). Therefore, VGluT2 was exclusively expressed by fully differentiated olfactory sensory neurons, once their axons have reached the corresponding targets at the glomeruli in the bulb.

VGluT2 protein expression in the Olfactory Epithelium

The mechanisms that regulate SV protein expression appear to be complex. Indeed, previous work has shown that, for some pre-synaptic molecules, there is a discordance between the patterns of mRNA expression and protein expression, suggesting that post-transcriptional mechanisms might be involved (Bergmann et al., 1991; Daly and Ziff, 1997; Lou and Bixby, 1993). Consequently we sought to determine whether VGluT2 protein was concomitantly expressed with its mRNA in the olfactory epithelium. Because VGluT2 is a synaptic vesicle membrane protein its main cellular localization is at the sensory neuron pre-synaptic terminals in the olfactory bulb. Nevertheless, we could detect VGluT2 protein in the cell body of olfactory sensory neurons, probably localized in the endoplasmic reticulum. VGluT2 protein overlapped with OMP protein in the olfactory epithelium (Figure 4I), suggesting that, at least in this olfactory tissue, there is no delay between the appearance of VGluT2 mRNA and immunocytochemically detectable VGluT2 protein. This also confirms that VGluT2 is restricted to mature OSNs, not only at the mRNA level but also at the protein level.

Expression of pre-synaptic molecules in target-deprived olfactory tissue

It has been reported that, both in chick ciliary ganglion and in spinal motor neurons, contact with the target regulates the expression of synaptotagmin 1 (Campagna et al., 1997; Plunkett et al., 1998). Therefore we investigated whether in mouse olfactory epithelium the expression of genes involved in synapse function was also dependent on contact between olfactory sensory neurons and second order neurons in the olfactory bulb. To address this question we performed unilateral olfactory bulbectomies (OBX), a surgical technique that involves the ablation of one of the olfactory bulbs, designed to deprive the ipsilateral sensory neurons of their target. Importantly, the contralateral epithelium, for which the olfactory bulb targets remain, is used as an internal control in this kind of experiment. It has been well documented that shortly after bulbectomy, contact-deprived sensory neurons become apoptotic and die causing a dramatic decrease in epithelial thickness on the experimental side of the epithelium that peaks at day 5 post-surgery (Carson et al., 2005; Cowan et al., 2001; Michel et al., 1994). Degeneration of the olfactory neurons after target deprivation is followed by an increase in basal cell proliferation, which gives rise to new neurons and leads to epithelial recovery. Nevertheless in the chronic absence of the bulb the epithelium remains thinner than normal and there is a significant reduction in the number of sensory neurons expressing OMP (Schwob et al., 1992; Verhaagen et al., 1990). We tested our panel of *in situ* hybridization probes in olfactory epithelium tissue from animals sacrificed 30 days post-bulbectomy (OBX-30), at which time the initial acute retrograde

degeneration has ceased and the epithelium has stabilized in thickness (Costanzo and Graziadei, 1983). We observed that the expression of all the pre-synaptic genes was restored after the tissue recovered from bullectomy (Figure 5). Furthermore, 30 days after surgery, VGluT2 expression was still restricted to OMP+ cells only (Figure 6), illustrating that the absence of the synaptic target did not alter sensory neuron pre-synaptic differentiation.

DISCUSSION

The present study was designed to correlate the pattern of expression of specific pre-synaptic molecules with the basic processes occurring during olfactory sensory neuron (OSN) maturation. *In situ* hybridization was used to reveal the localization of pre-synaptic mRNA molecules in the olfactory epithelium at different stages of the olfactory sensory neuron lineage. We observed that as olfactory sensory neurons mature they sequentially acquire the expression of pre-synaptic molecules (results are summarized in Figure 7). Thus, mRNA from genes encoding pre-synaptic molecules localized at the active zone (piccolo and bassoon) together with a T-SNARE (syntaxin 1A) and its interacting partner (Munc18-1) co-localized with MASH1 mRNA, a progenitor cell marker, illustrating the initial onset of pre-synaptic genes expression in the sensory neuron lineage. During the later stage of development, as determined by the expression of the immature sensory neuron marker GAP43, we observed expression of synaptic molecules associated with the synaptic vesicle membrane (the V-SNARE VAMP2, synapsin 1, synaptophysin and synaptotagmin 1) together with the T-SNARE SNAP25 and the trans-synaptic adhesion molecules α -neurexins. Finally, it is not until olfactory sensory neurons express OMP and are fully mature that Vesicle Glutamate Transporter 2 expression occurred, both at the mRNA and protein levels. We also assessed our panel of *in situ* hybridization probes in epithelial tissue from bullectomized mice. After recovery from surgery, the expression of all pre-synaptic molecules tested was restored; in particular VGluT2 mRNA and protein expression were still restricted to OMP+ cells after bullectomy. These results are consistent with the hypothesis that in the OE, maturation and pre-synaptic differentiation of olfactory sensory neurons can occur independently of their target.

To date little is known about how synapses between olfactory sensory neurons and second order neurons are formed, which pre-synaptic molecules are involved and how they are distributed along the olfactory receptor neuron lineage (Bergmann et al., 1993). Here we described that, as they mature, OSNs sequentially acquire the expression of specific pre-synaptic molecules. Interestingly, genes with the earliest onset of expression encode proteins that provide the structural basis to organize the release and retrieval of synaptic vesicles (SV), like piccolo and bassoon, and for proteins that are necessary for SV docking and fusion, such as syntaxin 1A and Munc18-1. Genes that are expressed later, when OSNs are still immature but already committed to a receptor neuron fate, encode for proteins known to promote synapse formation and assembly of the pre-synaptic secretory apparatus (α -neurexins), for proteins involved in exocytosis of vesicles (VAMP2 and SNAP25), for SV proteins implicated in the regulation of neurotransmitter release (synaptotagmin 1 and synapsin 1) and for synaptophysin, the most abundant SV membrane protein with still unclear function (Dean and Dresbach, 2006; Geppert et al., 1994; Missler et al., 2003; Rosahl et al., 1995; Sollner et al., 1993; Sudhof, 2004). The onset of expression of the pre-

synaptic genes described thus far precedes vesicular release of neurotransmitter. Although our experiments do not address when synaptic contact between OSNs and second order neurons is made under normal circumstances, the early expression of these genes may suggest other functions besides synapse formation. It has been proposed that exocytosis plays a fundamental role in axon outgrowth by allowing an increase of the surface of the plasma membrane (Futerman and Banker, 1996). Indeed treatments that disrupt membrane transport retard axon growth (Osen-Sand et al., 1993). Since most, if not all, of the genes with an early onset of expression studied here participate in exocytosis, we suggest that their early presence is involved not only in synapse formation but also in axon elongation.

The pre-synaptic gene with the latest onset of expression coincides with that of OMP expression and encodes VGluT2, a transporter involved in loading SVs with the neurotransmitter glutamate. We were unable to detect the other two glutamate transporter family members VGluT1 and VGluT3 mRNAs in the olfactory epithelium (data not shown). Consistent with that, it has been suggested that VGluT1 and VGluT3 proteins are absent from OSNs terminals in the olfactory bulb (Gabelle et al., 2007). Thus, in OSNs, vesicular release of neurotransmitter is likely dependent on VGluT2 exclusively and occurs only after cells are fully mature. Indeed vesicular uptake of glutamate is specific to glutamatergic axon terminals and is a necessary process before glutamate is released from synaptic vesicles into the synaptic cleft. Expression of VGluT2 in heterologous cells is sufficient to make gabaergic neurons glutamatergic (Takamori et al., 2001). Interestingly, VGluT2 protein is still detected in *omp* knock out background by immunohistochemistry (data not shown), suggesting that VGluT2 expression is not dependent on OMP. Also, it is of interest to note that similarly to VGluT2, Dopamine Receptor 2 mRNA is also absent from immature OSNs and is also present in fully mature OSNs only (Masayo Omura, Peter Mombaerts Lab, personal communication). This would suggest that OSNs acquire the ability to release glutamate concomitantly with the capacity of being modulated by dopamine.

In order to assess whether the expression of pre-synaptic molecules is activity dependent we tested our panel of *in situ* hybridization probes in olfactory epithelium tissue from animals lacking Adenylyl Cyclase 3 (Wong et al., 2000). In the knock out background all pre-synaptic molecules were expressed at the same stages in OSN lineage as in control tissue (unpublished observations). This result shows that the expression of pre-synaptic genes in the OE is not dependent on the cAMP-signaling cascade.

Regeneration of OSNs following recovery from bulbectomy has been studied extensively (Costanzo and Graziadei, 1983; Konzelmann et al., 1998; Shetty et al., 2005). Schwob et al., 1992 proposed that the olfactory bulb does not induce or stimulate neuronal differentiation but rather provides OSNs with the necessary trophic support for their survival (Schwob et al., 1992). Our results are in line with this hypothesis and show that pre-synaptic differentiation of OSNs can occur even in the absence of their post-synaptic partners. Altogether our results suggest that olfactory sensory neurons follow an intrinsic program of maturation and pre-synaptic differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Bassoon, piccolo, Munc18-1 and syntaxin 1A mRNA were expressed early in olfactory sensory neuron lineage.

Fluorescent double *in situ* hybridization was performed on coronal sections of post-natal day 5 mouse olfactory epithelium. RNA probes targeted against bassoon (A), piccolo (D), Munc18-1 (G) and syntaxin 1A (J) gene transcripts were combined individually with a probe directed against MASH1 mRNA (panels B, E, H and K respectively), a neuronal progenitor marker expressed by a subset of basal cells. The mRNA of these four genes co-localized

with MASH1 mRNA (panels C, F, I and L). Note that the expression of the four genes was not limited to MASH1+ cells only, but they continued to be expressed throughout the sensory neuron lineage. Scale bar: 30 μ m. Abbreviations: SC: Sustentacular Cells; OSNs: Olfactory Sensory Neurons; BC: Basal Cells.

A magenta-green copy of this figure is available as Supplementary Figure 3.

Figure 2.

Pan- α -neurexins, synapsin 1, VAMP2, SNAP25, synaptotagmin 1 and synaptophysin mRNA were expressed later in olfactory sensory neuron lineage.

Fluorescent double *in situ* hybridization was performed on post-natal day 5 mouse olfactory epithelium combining probes recognizing α -neurexins mRNA (A), synapsin 1 mRNA (D), VAMP2 mRNA (G), SNAP25 mRNA (J), synaptotagmin 1 mRNA (M) or synaptophysin mRNA (P) with a probe recognizing MASH1 mRNA (panels B, E, H, K, N and Q respectively). The expressions of the six pre-synaptic genes tested in this set of experiments

did not overlap with MASH1 expression (panels C, F, I, L, O and R). Pan-a-neurexin probe recognizes α -neurexin 1, 2 and 3 gene transcripts. Scale bar: 30 μ m. Abbreviations: SC: Sustentacular Cells; OSNs: Olfactory Sensory Neurons; BC: Basal Cells. A magenta-green copy of this figure is available as Supplementary Figure 4.

Figure 3.

Expression of pan- α -neurexins, synapsin 1, VAMP2, SNAP25, synaptotagmin 1 and synaptophysin mRNAs overlapped with that of GAP43 mRNA.

Fluorescent double *in situ* hybridization was performed on post-natal day 20 mouse olfactory epithelium. Probes recognizing α -neurexins mRNA (A), synapsin 1 mRNA (D), VAMP2 mRNA (G), SNAP25 mRNA (J), synaptotagmin 1 mRNA (M) or synaptophysin mRNA (P) were combined individually with a probe recognizing GAP43 mRNA (panels B, E, H, K, N and Q respectively). Immature GAP43+ cells did express these six pre-synaptic

genes, indicating that the expression of α -neurexins, synapsin 1, VAMP2, SNAP25, synaptotagmin 1 and synaptophysin begun during OSN maturation (panels C, F, I, L, O and R). Scale bar: 30 μ m. Abbreviations: SC: Sustentacular Cells; OSNs: Olfactory Sensory Neurons; BC: Basal Cells.

A magenta-green copy of this figure is available as Supplementary Figure 5.

Figure 4.

VGLUT2 was expressed by fully mature olfactory sensory neurons only.

(A, B and C) and (D, E and F): Fluorescent double *in situ* hybridization in post-natal day 20 mouse olfactory epithelium showing that Vesicle Glutamate Transporter 2 (VGLUT2) mRNA was expressed in cells that also expressed olfactory marker protein (OMP) and that VGLUT2 was absent from cells that express the immature cell marker GAP43. This indicated that the initiation of VGLUT2 expression coincided with that of OMP, a marker of mature olfactory sensory neurons.

(G, H and I): Immunohistochemistry in post-natal day 20 mouse olfactory epithelium showing co-localization of VGLUT2 and OMP at the protein level (anti-VGLUT2 in green, anti-OMP in red, nuclear marker TOTO-3 in blue). Area inside the dashed box in (I) is shown with higher magnification in the corresponding insets. Arrowheads indicate axon bundles stained for VGLUT2.

Scale bar: 30 μ m. Scale bar inset in (I): 10 μ m. Abbreviations: SC: Sustentacular Cells; OSNs: Olfactory Sensory Neurons; BC: Basal Cells.

A magenta-green copy of this figure is available as Supplementary Figure 6.

Figure 5.

Expression of pre-synaptic molecules by olfactory sensory neurons was restored after recovery from unilateral bullectomy.

Chromogenic *in situ* hybridization showing expression of OMP (a and b), bassoon (c and d), piccolo (e and f), Munc18-1 (g and h), syntaxin 1A (i and j), α -neurexins (k and l), synapsin 1 (m and n), VAMP2 (o and p), SNAP25 (q and r), synaptotagmin 1 (s and t) and synaptophysin (u and v) mRNAs in non-bullectomized (control) and bullectomized sides of mouse olfactory epithelium 30 days post-surgery. Note the thinning of the neuroepithelium

on the bulbectomized side when compared to the non-bulbectomized side. Scale bar: 50 μm . Abbreviations: control side: non-bulbectomized side of the olfactory epithelium; obx side: bulbectomized side of the olfactory epithelium.

Figure 6.

Expression of VGLUT2 was restored in olfactory neurons after recovery from unilateral bulbectomy.

(A, B, C, D, E and F): Fluorescent double *in situ* hybridization was performed combining probes for Vesicle Glutamate Transporter 2 (VGLUT2) and OMP in the bulbectomized side from olfactory epithelium 30 days post-bulbectomy. We observed that after recovery from bulbectomy VGLUT2 mRNA was still restricted to fully mature OMP+ neurons only.

(G, H, I, J, K and L): Immunohistochemistry showed co-localization of VGLUT2 and OMP proteins in the bulbectomized side of mouse olfactory epithelium 30 days post-surgery (anti-VGLUT2 in green, anti-OMP in red, nuclear marker TOTO-3 in blue).

(D, E, F) and (J, K, L) are higher magnification from areas inside the dashed boxes on (A, B, C) and (G, H, I) respectively. Scale bar in (A, B, C) and (G, H, I): 30 μ m, in (D, E, F) and (J, K, L): 10 μ m. Abbreviations: SC: Sustentacular Cells; OSNs: Olfactory Sensory Neurons; BC: Basal Cells.

A magenta-green copy of this figure is available as Supplementary Figure 7.

Figure 7.

Summary illustrating the function and distribution of the pre-synaptic molecules addressed in this study.

In situ hybridization was used in this study to reveal the localization of pre-synaptic mRNA molecules in the olfactory epithelium at different stages of the olfactory sensory neuron lineage. We observed that as olfactory sensory neurons mature they sequentially acquire the expression of pre-synaptic molecules. We observed that the pre-synaptic molecules bassoon, piccolo, syntaxin 1A and its interacting partner Munc18-1 (represented in blue) showed the earliest onset of expression during OSN lineage. The mRNA of these genes co-localized with MASH1 mRNA, a cellular marker for olfactory sensory neurons progenitors. The pre-synaptic molecules whose expression was initiated during OSN maturation are α -neurexins, synapsin 1, VAMP2, SNAP25, synaptotagmin 1 and synaptophysin (represented in orange). The expression of these genes overlapped with that of GAP43, a marker for immature olfactory sensory neurons. The pre-synaptic molecule with the latest onset of expression is

the Vesicle Glutamate Transporter 2 (represented in green). VGlut2 expression overlapped with OMP expression, a mature cellular marker, suggesting that is not until OSNs are fully mature that they acquire the ability to release glutamate. Neurexins and Munc18-1 interact indirectly through the CASK/Mint complex (Biederer and Sudhof, 2000). In turn, piccolo can interact with VAMP2 through the preanlylated Rab receptor protein PRA1 (Dresbach et al., 2001).

Table 1

mRNAs assessed by *in situ* hybridization. Abbreviation: ISH: *In Situ* Hybridization.

Probe Name	mRNA accession number	ISH probe nucleotides
Bassoon	NM_007567	11,381–12,037
gap43	NM_008083	531–1,146
munc18–1	NM_009295	2,654–3,181
Omp	NM_011010	1,373–2,012
pan- α -neurexin	NM_020252 (neurexin 1) NM_020253 (neurexin 2) NM_172544 (neurexin 3)	1,924–2,611 901–1,595 337–1,1013
piccolo	NM_011995	15,204–16,029
snap25	NM_011428	36–574
synapsin 1	BC022954	2,367–3,143
synaptophysin	NM_009305	697–1,602
synaptotagmin1	NM_009306	2,999–3,503
syntaxin1a	NM_016801	1,046–1,586
vamp2	NM_009497	1,438–2,110
VGlut2	BC038375	3,353–3,728