



Differential regulation of neurexin at glutamatergic and GABAergic synapses

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Neurexins (Nrxs) have emerged as potential determinants of synaptic specificity, but little is known about their localization at central synapses. Here we show that Nrxs have a remarkably selective localization at distinct types of glutamatergic synapses and we reveal an unexpected ontogenetic regulation of Nrx expression at GABAergic synapses. Our data indicate that synapses are specified by molecular interactions that involve both Nrx-dependent and Nrx-independent mechanisms. We propose that differences in the spatio-temporal profile of Nrx expression may contribute to specify the molecular identity of synapses.

Keywords: neurexin, neuroligin, GABA_A receptor, synaptic specificity, cerebellum

INTRODUCTION

The assembly, validation, and specificity of synapses are thought to depend on *trans*-synaptic interactions between cell-adhesion molecules (Waites et al., 2005; Shen and Scheiffele, 2010; Williams et al., 2010). Neurexins (Nrxs) are among the best characterized adhesion molecules that have been implicated in synapse formation and synaptic specificity (Ichtchenko et al., 1995; Craig and Kang, 2007). When expressed in non-neuronal cells, Nrxs induce synapse formation on co-cultured neurons, suggesting that they function in the initial assembly of synaptic connections (Graf et al., 2004; Nam and Chen, 2005). Deletion of α -Nrxs in mice results in a lethal phenotype characterized by a massive impairment in Ca²⁺-channel function and neurotransmitter release (Missler et al., 2003; Zhang et al., 2005). In *Drosophila*, mutation of the single Nrx gene causes severe structural defects of the neuromuscular junction, and corresponding alterations in synaptic transmission (Li et al., 2007). These genetic data suggest that although Nrxs may not be strictly required for synaptogenesis, they are crucial for the proper assembly and functional maturation of synapses. Accordingly, human genetic studies have evidenced that mutations in Nrx genes are linked to several psychiatric disorders, including autism and schizophrenia (reviewed in Südhof, 2008; Betancur et al., 2009).

There are three Nrx genes in mammals, each encoding α and β transcripts, that are further subject to alternative splicing resulting in potentially thousands of different isoforms (Ullrich et al., 1995). These molecules bind to multiple, structurally diverse postsynaptic partners. In addition to neuroligins, that were the first characterized Nrx-binding partners, Nrxs bind to a variety of other molecules, including leucine-rich repeat transmembrane proteins (LRRTMs), neurexophilins, dystroglycan, GABA_A receptors (GABA_ARs), and cerebellin-1 (Cbln1; for review, see Siddiqui

and Craig, 2011). These interactions are regulated at the level of mRNA alternative splicing, generating a complex molecular “code” that may be important for synapse specification (Boucard et al., 2005; Chih et al., 2006; Uemura et al., 2010). In vertebrates, Nrxs are synthesized throughout the brain in all excitatory and inhibitory neurons (Ullrich et al., 1995). Therefore, it is generally assumed that they have a general role in synapse development and act as presynaptic hub molecules that mediate synapse maturation via selective interactions with different ligands (Südhof, 2008; Siddiqui and Craig, 2011). However, the distribution of endogenous Nrxs at distinct types of excitatory and inhibitory synapses is largely unknown, due to the lack of antibodies suitable for immunohistochemistry.

In this study, we used a pan-Nrx antiserum raised against a conserved intracellular region. Using the cerebellum as a model system, we show that Nrxs have a selective localization at distinct types of glutamatergic synapses. We also demonstrate that GABAergic interneurons regulate the synaptic expression of Nrxs during postnatal development. These differences in localization suggest that Nrxs have distinct roles in the development of glutamatergic and GABAergic synapses.

RESULTS

NEUREXINS ARE PRESENT AT MOST BUT NOT ALL CEREBELLAR SYNAPSES

We tested the rabbit pan-Nrx antiserum on cerebellar sections processed with different fixation techniques. We found that Nrx epitopes were sensitive to prolonged aldehyde fixation, and weak fixation protocols were therefore used to visualize Nrx immunoreactivity (see Materials and Methods). Nrx labeling was punctate, supporting synaptic localization (**Figure 1**). Moreover, double labeling with another pan-Nrx antibody raised in chicken

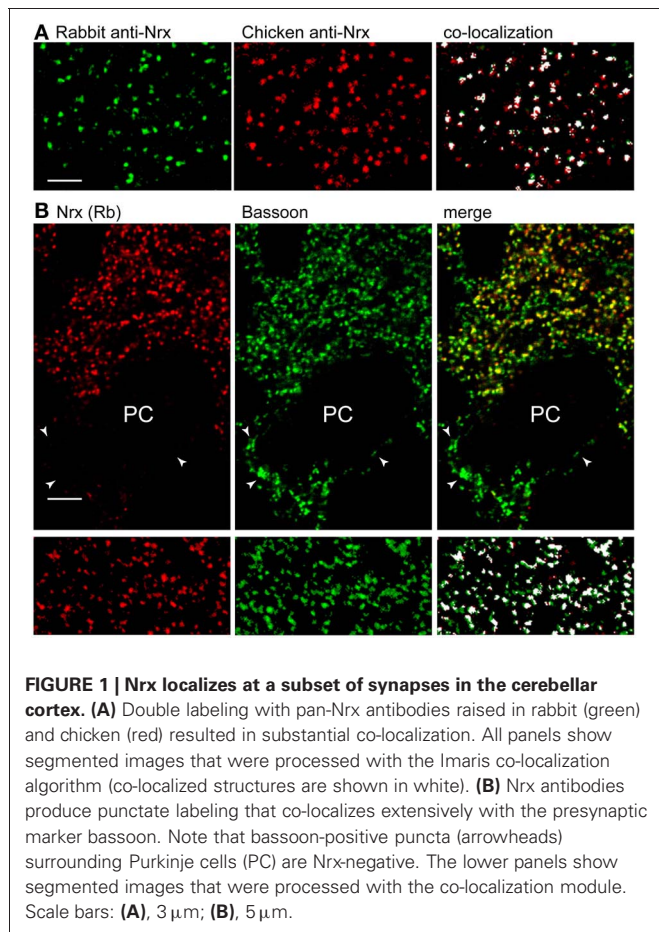


FIGURE 1 | Nrx localizes at a subset of synapses in the cerebellar cortex. (A) Double labeling with pan-Nrx antibodies raised in rabbit (green) and chicken (red) resulted in substantial co-localization. All panels show segmented images that were processed with the Imaris co-localization algorithm (co-localized structures are shown in white). (B) Nrx antibodies produce punctate labeling that co-localizes extensively with the presynaptic marker bassoon. Note that bassoon-positive puncta (arrowheads) surrounding Purkinje cells (PC) are Nrx-negative. The lower panels show segmented images that were processed with the co-localization module. Scale bars: (A), 3 μm ; (B), 5 μm .

(Dean et al., 2003) revealed extensive co-localization (Figure 1A), indicating that both antibodies recognize the same molecules. However, the rabbit antiserum produced a stronger labeling and was therefore used in all subsequent experiments.

To establish synaptic localization, we performed double immunofluorescence for Nrx and the presynaptic matrix protein bassoon (Figure 1B). Quantitative analysis of co-localization revealed that practically all Nrx-positive puncta in the molecular layer were associated with bassoon. However, Nrx was present in $\sim 80\%$ of bassoon-positive synapses (4204 co-localized puncta out of a total of 5109 bassoon-positive puncta in two different cerebella), implying that some cerebellar synapses lack Nrx. In particular, perisomatic synapses surrounding PCs were Nrx-negative, suggesting that Nrx may be absent from GABA synapses (see below).

NEUREXINS ARE PRESENT AT PF BUT NOT CF SYNAPSES

The cerebellum provides an exquisite example of synaptic specificity. Purkinje cells (PCs), the principal neurons of the cerebellar cortex, receive glutamatergic input from two distinct sources: parallel fibers (PFs) establish synapses with spines located on the distal dendrites, whereas climbing fibers (CFs) contact spines located in the proximal dendritic domain (Yuste and Bonhoeffer, 2004; Cesa and Strata, 2009). Confocal imaging in L7-GFP mice showed that Nrx puncta were closely associated with PC spines,

suggesting localization in PFs (Figure 2A). This was confirmed by immunogold labeling, showing gold particles at the presynaptic side of PF-PC synapses (Figure 2B). In contrast, we did not find Nrx signals in CF terminals, labeled with antibodies against the vesicular glutamate transporter VGLUT2, indicating that in the cerebellar cortex Nrx is selectively localized at specific types of glutamatergic synapses (Figure 2C).

The presence of Nrxs at PF-PC synapses was further evidenced in double-labeling with the metabotropic glutamate receptor mGluR1a, that labels selectively PC spines (Tanaka et al., 2000). These experiments revealed that Nrx puncta were clustered opposite to mGluR1a-positive spines (Figure 2D). Notably, the pattern of Nrx expression in PF terminals was severely disrupted in mutant *hotfoot* mice, that lack the glutamate receptor GluRD2 (Mandolesi et al., 2009). In these animals, the size of Nrx puncta was significantly decreased (Kolmogorov-Smirnov, $p < 0.001$, $n = 3$) compared to the control situation (Figure 2F), as also documented by the presence of numerous small puncta (generally $< 0.04 \mu\text{m}^2$) characterized by considerably reduced brightness and fuzzy appearance (Figure 2E). These small puncta were generally not associated with PC spines (Figure 2E), suggesting that Nrx fails to form stable clusters when PFs are not connected to the appropriate postsynaptic targets. Moreover, the density of the larger Nrx puncta ($0.04\text{--}0.2 \mu\text{m}^2$) was significantly reduced in the *hotfoot* cerebellum (mean \pm SEM puncta/1000 μm^2 : control, 473.5 ± 39 ; *hotfoot*, 303 ± 19 ; $n = 6$ sampling fields from 3 mice; unpaired *t*-test, $P = 0.0061$), consistent with the robust reduction of synapses between PFs and PCs reported previously (Mandolesi et al., 2009). These data are in agreement with the idea that the integrity of PF synapses depends on a tripartite *trans*-synaptic complex comprising Nrx, the secreted glycoprotein Cbln1 and GluD2 (Matsuda et al., 2010; Uemura et al., 2010).

NEUREXINS ARE MAINLY ASSOCIATED WITH DEVELOPING GABA SYNAPSES

We next analyzed the expression of Nrxs at GABAergic synapses. In the mature cerebellum ($>P30$), Nrx was below detectable levels at inhibitory synapses labeled for the GABA_A $\alpha 1$ subunit (Figure 3A) or the scaffold molecule gephyrin (not shown). Quantitative evaluation of co-distribution in the molecular layer revealed that $\sim 3\%$ of GABA synapses were Nrx-positive (162 co-distributed puncta out of a total of 5236 GABA_AR-positive puncta). However, in postnatal mice (P5–P21) Nrx was found in practically all GABA synapses (co-distribution index at P15: 97%, 2810 co-distributed puncta out of a total of 2901 GABA_AR-positive puncta), including perisomatic and axo-dendritic synapses made by basket cells and stellate cells (Figure 3B).

To validate these immunohistochemical findings, we isolated synaptosomes from cerebella of P15 and adult ($>P30$) mice, and we evaluated the potential of neuroligin-2 (NL2) to co-immunoprecipitate Nrx from developing and mature synapses. Because NL2 is present at GABA synapses since early stages of synaptogenesis (Patrizi et al., 2008), it can be effectively used to monitor developmental changes in the expression of interacting proteins. As expected, co-immunoprecipitation of NL2 from synaptosomes yielded considerably higher Nrx levels in juvenile

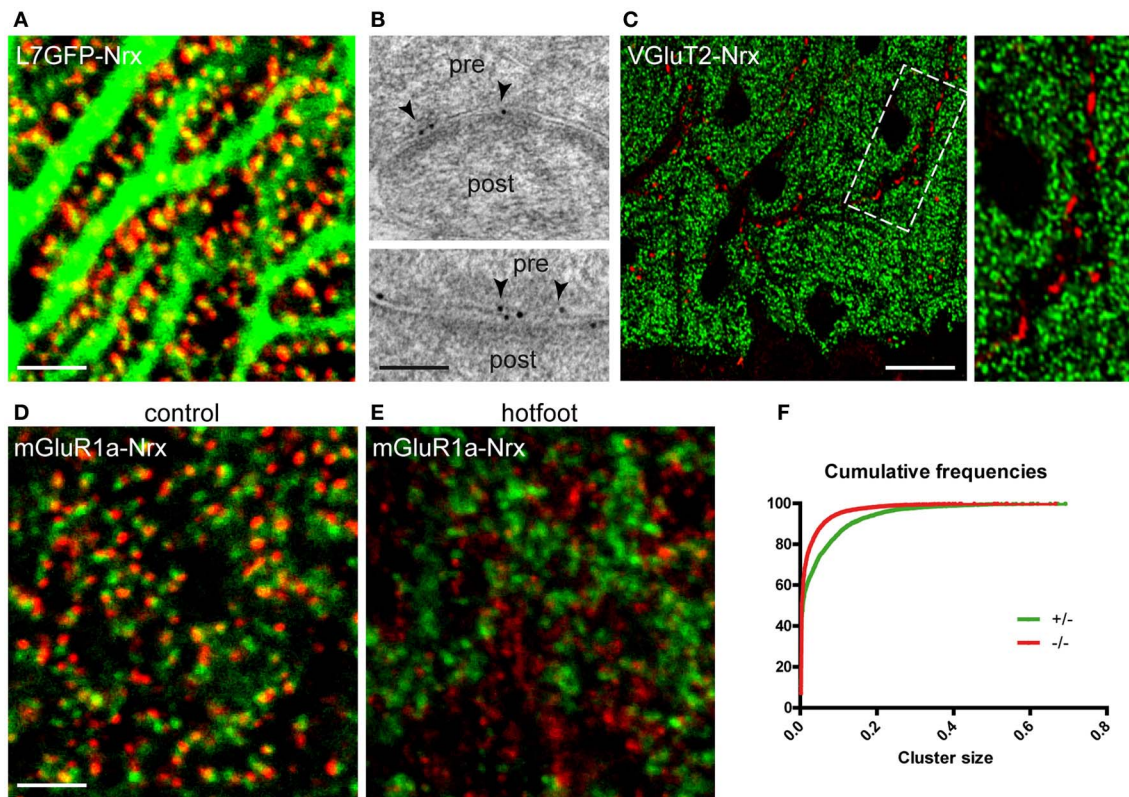


FIGURE 2 | Selective localization of Nrxa at synapses made by PFs but not CFs. (A) Nrxa puncta (red) are closely apposed to Purkinje cell spines in a L7-GFP mouse cerebellum. **(B)** Immunogold labeling (arrowheads) reveals the presence of Nrxa at synapses between parallel fibers (pre) and Purkinje cell spines (post). **(C)** Nrxa (green) is not associated with VGlut2-positive climbing fiber terminals (red). The boxed area is shown at higher magnification in the inset. **(D,E)** Double labeling for Nrxa (red) and mGluR1a (green) in the cerebellum of control **(D)** and *hotfoot* **(E)** mice. Note the altered pattern of Nrxa localization in the *hotfoot* mutant. **(F)** Cumulative plot showing the reduced size of Nrxa puncta in *hotfoot* mice compared to control. Scale bars: **(A,D,E)**, 3 μm ; **(B)**, 100 nm; **(C)**, 18 μm .

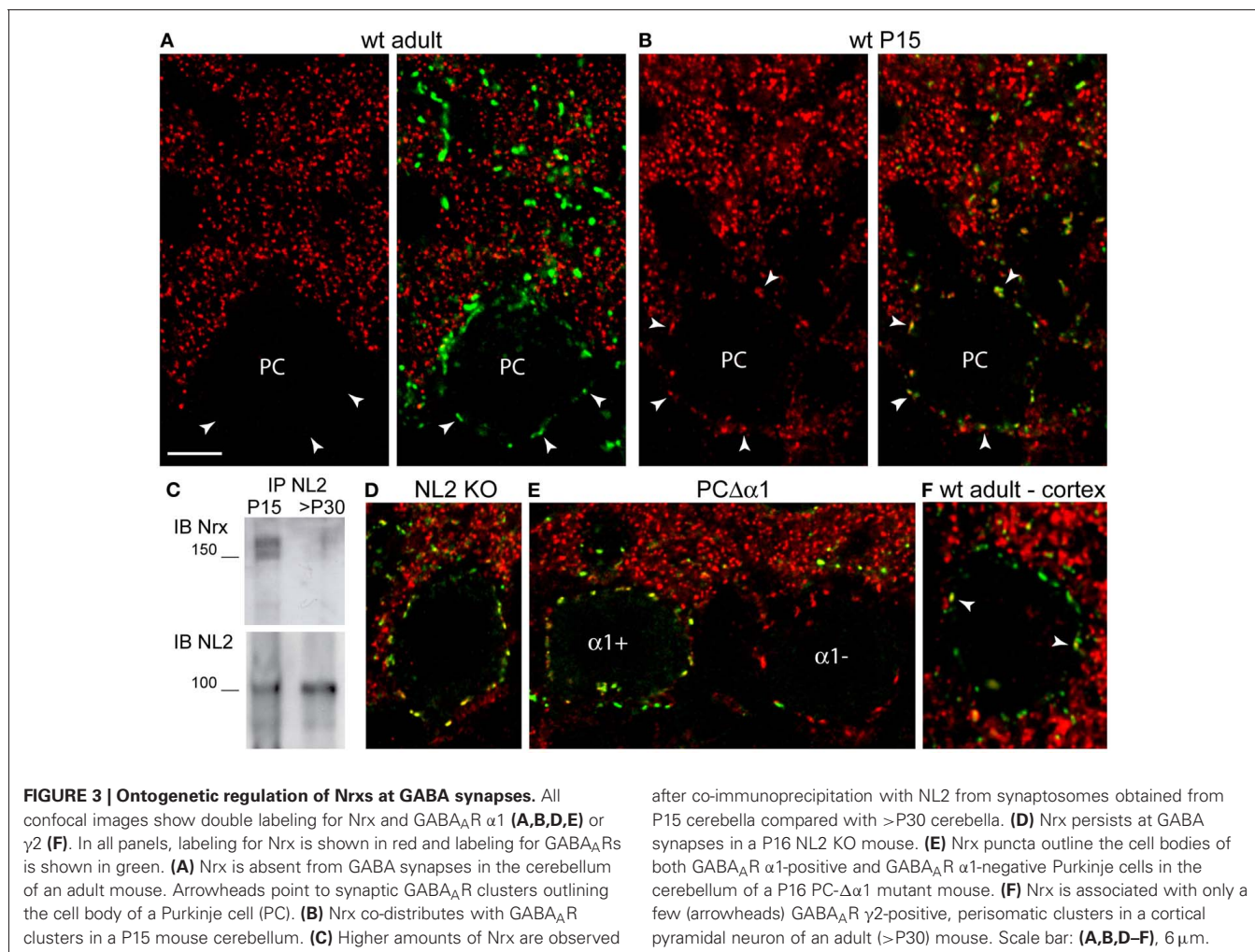
than in adult mice (**Figure 3C**), supporting the idea that Nrxa is present in developing GABA synapses, but is downregulated at the end of the synaptogenic period.

We examined whether the expression of Nrxa at developing GABA synapses depends on NL2 or GABA_ARs, that are Nrxa-binding partners at inhibitory synapses (Graf et al., 2004; Varoqueaux et al., 2004; Zhang et al., 2010). We found no defects in synaptic localization of Nrxa in P16 NL2 KO mice (**Figure 3D**; mean \pm SEM Nrxa clusters/50 μm of perisomatic membrane: WT, 23.5 ± 0.45 , $n = 44$ cells; KO, 24.3 ± 0.36 , $n = 57$ cells; unpaired t -test, $p = 0.1637$). We then analyzed PC- $\Delta\alpha 1$ mice, that have a selective deletion of the GABA_AR $\alpha 1$ subunit gene in PCs, resulting in complete loss of GABA_ARs (Briatore et al., 2010). Deletion of the $\alpha 1$ subunit is asynchronous among different PCs, resulting in a characteristic mosaic-like pattern, with $\alpha 1$ -positive and $\alpha 1$ -negative cells, in P14–P16 mice (**Figure 3E**). We found no differences in the synaptic localization of Nrxa in $\alpha 1$ -positive and $\alpha 1$ -negative PCs of P16 PC- $\Delta\alpha 1$ mice (**Figure 3E**; mean \pm SEM Nrxa clusters/50 μm of somatic membrane: $\alpha 1$ -positive PCs, 19.9 ± 0.64 , $n = 33$ cells; $\alpha 1$ -negative PCs, 19.6 ± 0.46 , $n = 32$ cells; unpaired t -test, $p = 0.7365$). These results suggest that neither NL2 nor GABA_ARs are essential for Nrxa localization at developing GABAergic synapses.

To establish whether the transient expression of Nrxa is a general feature of GABA synapses, we analyzed co-distribution with the obligatory $\gamma 2$ subunit of synaptic GABA_ARs in sensorimotor cortex of adult mice (**Figure 3F**). We found that in adult animals Nrxa was associated with a small percentage of GABAergic synapses labeled with antibodies against the GABA_AR $\gamma 2$ subunit. Quantification in pyramidal neurons ($n = 28$ cells from four mice) revealed that $\sim 30\%$ of perisomatic synapses were Nrxa-positive. Unfortunately, labeling for Nrxa was generally quite challenging outside of the cerebellum, especially when combined with other antibodies, which precluded a detailed characterization of the Nrxa-positive synapses. For the same reason, we could not analyze Nrxa localization at developing synapses in postnatal mice. However, the limited co-distribution with GABA_ARs substantiates the idea that Nrxa are scarcely represented at mature GABAergic synapses.

DISCUSSION

The three mammalian Nrxa genes undergo extensive alternative splicing in their extracellular domain, potentially generating thousands of different isoforms (Ullrich et al., 1995). Nrxa are widespread in neurons, which has led to the general assumption that Nrxa isoforms could determine synaptic properties



by interacting selectively with specific postsynaptic partners (Südhof, 2008; Williams et al., 2010; Siddiqui and Craig, 2011). Our present findings add a new dimension to the concept of a “molecular synaptic code” (Selimi et al., 2009), by showing that Nrnx undergo differential spatio-temporal regulation at distinct types of glutamatergic and GABAergic synapses.

We report two principal results. First, we show that Nrnx have a remarkably selective localization in PFs but not in CFs of the cerebellar cortex (**Figures 2A–C**). These afferents innervate distinct domains of the PC dendritic arbor, a process that depends on activity-dependent competition, as well as on differences in their molecular organization (Cesa and Strata, 2009; Kano and Hashimoto, 2009; Yuzaki, 2011). Our observations agree well with recent data showing that Nrnx acts as a presynaptic ligand for the GluD2-Cbln1 complex at PF synapses (Matsuda et al., 2010; Uemura et al., 2010). Extensive evidence indicates that Cbln1, which is secreted by PFs, acts as a bi-directional receptor for both GluD2 and Nrnx, thus providing a physical linkage between the postsynaptic density in PC spines and the active zone in PF terminals (Uemura et al., 2010; Yuzaki, 2011). Consistent with this idea, we found that Nrnx clusters were altered in the cerebellum of *hotfoot* mice (**Figure 2E**), that lack GluD2. First,

there was a significant reduction in the density of the larger Nrnx puncta, that presumably corresponded to synapses between PFs and PCs. Second, many small clusters unrelated to PC spines became apparent in the molecular layer, suggesting that the presynaptic localization of Nrnx depends on the structural integrity of PF synapses and proper molecular interactions mediated by the GluD2-Cbln1 complex. On a more general level, the selective localization of Nrnx at PF but not CF synapses indicates that these glutamatergic contacts depend on *trans*-synaptic interactions mediated by distinct complexes of adhesion molecules. While the Nrnx/Cbln1/GluD2 triad likely represents a “molecular code” for PF synapses (Uemura et al., 2010), the identity of adhesion molecules expressed at CF synapses remains to be determined. Synaptic protein profiling (Selimi et al., 2009) and other proteomic strategies may help to decipher signaling pathways that are selectively involved in the specification of these glutamatergic contacts.

Another important finding was that Nrnx undergo a developmentally regulated expression at GABA synapses. The situation in the cerebellar cortex is paradigmatic. Here Nrnx was associated with GABA synapses during the entire period of postnatal development, but was strongly downregulated at the end of

synaptogenesis, resulting in undetectable levels in mature circuits (Figures 3A,B). This transient expression is in perfect agreement with *in vitro* analyses, showing that overexpression of Nrxs in cultured neurons did not impair existing GABA synapses, but affected selectively the properties of developing contacts (Zhang et al., 2010). Together, these data suggest that Nr_x may act to regulate the functional maturation of developing GABAergic synapses. Interestingly, a recent study in *Caenorhabditis* showed that Nr_x and NL mediate a retrograde synaptic signal that inhibits neurotransmitter release by adjusting the rate and duration of synaptic vesicle release (Hu et al., 2012). Studies on cultured neurons also have demonstrated that Nr_xs suppress GABAergic synaptic transmission by direct binding to GABA_ARs (Zhang et al., 2010). Therefore, we speculate that the ontogenetically regulated expression of Nr_x at GABA synapses may represent a mechanism to adjust the levels of transmission at a time when GABA has a strong influence over the developmental assembly of neuronal circuits (Akerman and Cline, 2007; Huang and Scheiffele, 2008; Wang and Kriegstein, 2009).

The expression profile in neocortex was quite different, as Nr_x was retained in a subset of GABA synapses in adult mice (Figure 3F). It is of note that Nr_xs bind directly to α 1-GABA_ARs (Zhang et al., 2010), that are the unique type of GABA_AR in PCs and have a more limited distribution in neocortical neurons. It is possible that Nr_xs may be differently regulated at distinct types of inhibitory synapses characterized by different GABA_AR subtypes. Unfortunately, we have been unable to address this question due to the difficulties in detecting endogenous Nr_xs. It should also be mentioned that the pan-Nr_x antiserum used in this study was raised against a C-terminal amino acid sequence of Nr_x1 that is largely conserved in the Nr_x2 and Nr_x3 isoforms (see Materials and Methods). While immunoblot analyses have shown that the antiserum recognizes all Nr_x isoforms (Bottos et al., 2009), we cannot exclude that our observations are biased by higher affinity of the antiserum for Nr_x1 than for the other Nr_xs. The situation is also complicated by the fact that other than the original *in situ* hybridization mapping, that revealed differential but overlapping distribution of Nr_x isoforms in distinct classes of neurons (Ullrich et al., 1995), very little has become available about the expression of the different isoforms and their regulation at the cellular and synaptic levels.

Our current results suggest that the localization of Nr_x at developing GABA synapses is not influenced by binding to its postsynaptic ligands NL2 and GABA_ARs (Figures 3D,E). However, our observations in NL2 KO mice may be confounded by compensatory changes involving other NL isoforms. Indeed, we have observed a strong upregulation of NL3 and NL4 in PCs of NL2 KO mice (data not shown). Moreover, cellular imaging after artificial expression of tagged Nr_x isoforms in cortical interneurons demonstrated that the presynaptic localization of Nr_x1 β depends on binding to postsynaptic ligands (Fu and Huang, 2010). On the other hand, no GABA_ARs are present in PCs of PC- $\Delta\alpha$ 1 mice (Briatore et al., 2010), suggesting that binding to GABA_ARs is not essential for presynaptic stabilization of Nr_xs.

While there is no doubt that endogenous Nr_xs are presynaptic, it has been proposed that these adhesion molecules may partly

localize postsynaptically (Taniguchi et al., 2007). Unfortunately, postembedding electron microscopy yielded relatively weak labeling, precluding quantitative analysis of gold particle distribution at PF-PC synapses. However, the precise apposition of NRX-positive puncta with PC spines evidenced by confocal microscopy (Figure 2D) is consistent with a predominantly presynaptic localization.

In conclusion, our findings suggest that synapses are specified by molecular interactions that involve both Nr_x-dependent and Nr_x-independent mechanisms. Moreover, the complex spatio-temporal expression profile reported here suggests that ontogenetic regulation of Nr_xs is of crucial importance for determining how synapses form and acquire their functional maturation.

MATERIALS AND METHODS

MICE

Most of the experiments described in this study were performed on WT mice of the C57BL/6 strain and transgenic mice expressing GFP selectively in PCs (L7-GFP mice). In addition, three lines of mutant mice were used. *Hotfoot* mice are spontaneous mutants with a deletion of the glutamate receptor GluRD2 (Lalouette et al., 1998). GluD2 is expressed selectively in PC spines innervated by PFs and is crucial for the formation and maintenance of PF synapses (Kashiwabuchi et al., 1995; Yuzaki, 2003). Like GluRD2 knockout (KO) mice, *hotfoot* mutants have a reduced number of synapses between PFs and PCs (Mandolesi et al., 2009). The generation of PC- $\Delta\alpha$ 1 mice has been described elsewhere (Briatore et al., 2010). Deletion of the GABA_AR α 1 subunit gene was driven by Cre recombinase expressed under control of the L7 promoter, which resulted in a characteristic mosaic pattern, with α 1-positive and α 1-negative cells, in P14–P16 mice (Figure 3E). Finally, NL2 KO mice (Varoquaux et al., 2006) were kindly provided by Nils Brose (Max-Planck-Institute of Experimental Medicine and Center for Molecular Physiology of the Brain, Göttingen, Germany).

The experimental procedures were designed in accordance with national (Legislative Decree 116/92 and law n. 413/1993) and international (Directive 86/609/EEC and the recommendation 2007/526/EC from the European Community) laws and policies, and approved by the Italian Ministry of Health (Department of Public Veterinary Health) and by the ethical committee of Turin University.

ANTIBODIES

We used an affinity-purified, pan-Nr_x antiserum produced in rabbits as described in detail elsewhere (Bottos et al., 2009). This antibody was raised against the peptide AKSANKNKKNKDKEYV located in the intracellular region of the α and β isoforms of mouse Nr_x1. This region has high homology to Nr_x2 (PKTPSKAKKNKDKEYV) and Nr_x3 (SKSGHKKQKNKDKEYV). Consistent with this, the antiserum recognized all Nr_x isoforms in immunoblotting of transfected HeLa cells. Another antiserum (kindly provided by Peter Scheiffele, Biozentrum, Basel, Switzerland) was raised in chicken against a recombinant neurexin-GST fusion protein containing the cytoplasmic tail of Nr_x1 (Dean et al., 2003).

This antiserum recognized two bands with apparent molecular weights corresponding to α and β Nrxs in western blots of total cerebellar lysates. A rabbit antiserum against NL2 was obtained from Synaptic Systems (cat. no. 129203). Guinea pig antisera against the vesicular glutamate transporter VGLUT2 (Chemicon, AB2251) and the metabotropic glutamate receptor mGluR1a (kindly provided by Masahiko Watanabe, Hokkaido University, Sapporo, Japan) were used to label respectively CF terminals and PC spines (Tanaka et al., 2000; Fremeau et al., 2001). GABA synapses were identified with guinea pig antibodies against the $\alpha 1$ and $\gamma 2$ subunits of GABA_ARs (kindly provided by Jean-Marc Fritschy, University of Zurich, Switzerland). The monoclonal antibody against bassoon was obtained from Stressgen (cat. no. VAM-PS003).

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Detection of Nrx required a brief fixation protocol described in detail elsewhere (protocol B in Schneider Gasser et al., 2006; see also Patrizi et al., 2008). In some cases, mice were perfused with 2% formaldehyde, and the brains were postfixed in the same fixative overnight. This fixation protocol allowed simultaneous detection of Nrx and VGLUT2. Confocal images were acquired with a laser scanning confocal microscope (Zeiss LSM5 Pascal) using the multitrack mode to avoid fluorescence crosstalk. Synaptic puncta were analyzed on images acquired with a $\times 100$ oil-immersion objective (1.4 NA) at a magnification of $8.1 \times 10^{-3} \mu\text{m}^2/\text{pixel}$, and the pinhole set at 1 Airy unit. The images were processed with the image-analysis program Imaris (release 4.2; Bitplane). Clusters were quantified on segmented images with NIH Image J software. Co-localization was estimated on segmented images with the Imaris co-localization module (see Viltono et al., 2008). The density of perisomatic clusters was determined by counting manually Nrx puncta surrounding the cell body of PCs.

IMMUNOGOLD LABELING

Juvenile (P17) mice ($n = 2$) were perfused with 2% formaldehyde and 0.1% glutaraldehyde in sodium acetate buffer (pH 6) for 2 min, followed by 1 h perfusion with 2% formaldehyde and 0.1% glutaraldehyde in 0.1 M borate buffer (pH 9). Brains were postfixed in the second fixative solution overnight. Tissue blocks from the cerebellar vermis were freeze-substituted and embedded in Lowicryl HM20. Ultrathin sections were processed for the immunogold method using as secondary antibodies goat Fab fragments coupled to 10 nm colloidal gold particles (Sassoè-Pognetto and Ottersen, 2000).

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SYNAPTOSOMAL PREPARATION AND CO-IMMUNOPRECIPITATION

Synaptosome extraction was performed using the Syn-PER Reagent (Thermo Scientific) following the manufacturer's protocol. Frozen cerebella obtained from P15 and adult ($>P30$) mice were disaggregated with a tissue potter and lysed on ice with Syn-PER Reagent (10 ml per gram of tissue) added with protease and phosphatase inhibitors (50 $\mu\text{g}/\text{mL}$ pepstatin, 50 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 mM PMSE, 100 μM ZnCl₂, 1 mM Na orthovanadate, and 10 mM NaF). The solution was centrifuged at 1200 g for 10 min at 4°C, the pellet was discarded and the remaining supernatant was centrifuged at 10,000 g for 45 min at 4°C. The resulting pellet was resuspended in 2 ml of lysis buffer [10 mM Tris HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA, pH 8; 10% glycerol; 1% Triton X-100; and 1% 3-[(3-Cholamidopropyl)dimethylammonio]1-propanesulfonate (CHAPS)] with protease and phosphatase inhibitors (50 $\mu\text{g}/\text{mL}$ pepstatin, 50 $\mu\text{g}/\text{mL}$ leupeptin, 10 $\mu\text{g}/\text{mL}$ aprotinin, 1 mM PMSE, 100 μM ZnCl₂, 1 mM Na orthovanadate, and 10 mM NaF). After quantification with the BCA Protein Assay Reagent Kit (Pierce Chemical Co.), samples (5 mg of total proteins) were precleared with protein A-Sepharose (Amersham Biosciences) and incubated 1 h with the rabbit anti-Nrx antibody (1.5 $\mu\text{g}/\text{mg}$) or the rabbit anti-NL2 antibody (2.5 $\mu\text{g}/\text{mg}$). Immune complexes were recovered on protein A-Sepharose overnight and washed 4 times. Proteins were separated by 4–15% SDS/PAGE electrophoresis gel, transferred to polyvinylidene difluoride membrane (Millipore), and detected by immunoblot. Immunoreactive proteins were identified with an HRP-conjugated secondary antibody (Jackson ImmunoResearch) and visualized by an ECL system (Amersham Biosciences).

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