Journal of Chemical Neuroanatomy 36 (2008) 160–169

Contents lists available at ScienceDirect

Journal of Chemical Neuroanatomy

journal homepage: www.elsevier.com/locate/jchemneu

Olfactory bulb interneurons releasing NO exhibit the Reelin receptor ApoEr2 and part of those targeted by NO express Reelin

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ARTICLE INFO

Article history:
Received 4 March 2008
Received in revised form 12 August 2008
Accepted 13 August 2008
Available online 30 August 2008

Keywords:
Reelin
ApoEr2
GC
nNOS
Periglomerular cells
Granule cells
Olfactory

ABSTRACT

Nitric oxide (NO) and Reelin both modulate neuronal plasticity in developing and mature synaptic networks. We recently showed a loss of neuronal nitric oxide synthase (nNOS) protein in the olfactory bulb of reeler mutants and advanced the hypothesis that the Reelin and NO signalling pathways may influence each other. We now studied the distribution of NO sensitive guanylyl cyclase (NOsGC), Reelin and its receptor Apolipoprotein E2 (ApoEr2) in the olfactory bulb by multiple fluorescence labelling and tested whether nNOS and ApoEr2 colocalize in this area. We also essayed the protein content of NOsGC in the reeler olfactory bulb and tested whether there are any changes in nNOS and NOsGC protein in other reeler brain areas.

Olfactory bulb interneurons expressing ApoEr2 and nNOS are only few in the glomerular layer but represent the large majority of granule cell layer interneurons. Conversely, NOsGC interneurons are rare in the granule cell layer and abundant as periglomerular cells. Reelin containing periglomerular cells almost entirely belong to the NOsGC subset. These data further support the hypothesis of a reciprocal signalling between Reelin/NOsGC and ApoEr2/nNOS expressing neurons to affect olfactory bulb activity.

We also show that a significant rise in NOsGC content accompanies the decrease of nNOS protein in the reeler olfactory bulb. The same reciprocal changes present in the cortex/striatum and the hippocampus of reeler mice. Thus, the influence that the deficit of extracellular Reelin seems to exert on nNOS and its receptor is not limited to the olfactory bulb but is a general feature of the reeler brain.

1. Introduction

Nitric oxide (NO) contributes to the formation of axonal projections during development (Trimm and Rehder, 2004) and sustains synaptic plasticity in the adult nervous system (Garthwaite, 2005). NO is produced in neurons by the brain isoform of neuronal nitric oxide synthase (nNOSα). nNOSα contains a PDZ-domain enabling its association with the post-synaptic density-95 protein (PSD-95), the scaffolding protein of NMDA receptors. nNOS must associate at synapses to couple NO synthesis with glutamatergic activity. Ca^{2+} influx through the NMDA receptors channel results in calmodulin activation which in turn activates nNOS (Garthwaite, 1995). NO targets the heterodimeric hem proteins nitric oxide sensitive guanylyl cyclases (NOsGC referred to as GC in the text). These proteins consist of two subunits. Each subunit exists in two isoforms (α_1/α_2 and β_1/β_2) and the most expressed isoforms in the brain are the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ (Gibb and Garthwaite, 2001). Differences in the α subunit composition influence the intracellular distribution of GC. The α_2 subunit binds to PDZ-domains and allows the GC to associate with the NMDA receptor complex as well (Russwurm et al., 2001; Russwurm and Koesling, 2004). GC stimulates the production of cyclic 3′,5′-guanosine monophosphate (cGMP) which, in turn, activates cGMP dependent protein kinases, phosphodiesterases and cyclic nucleotide gated channels (Lucas et al., 2000).

Reelin contributes to the correct development of laminated structures (D'Arcangelo, 2006), promotes postnatal neuronal maturation of synaptic networks (Sinagra et al., 2005) and affects synaptic activity in the adult hippocampus (Chen et al., 2005). The very low density lipoprotein receptor (VLDLr) and the Apolipoprotein E receptor 2 (ApoEr2) represent the main Reelin receptors. The canonical Reelin signal requires Dab1 recruitment to these

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Abbreviations: ApoE, Apolipoprotein E; ApoEr2, Apolipoprotein E2 receptor; CB, calbindin; cGMP, cyclic guanosine monophosphate; CR, calretinin; DCX, doublecortin; GABA, γ-amino-butyric acid; GAD, glutamic acid decarboxylase; GCβ1, β1 subunit of guanylyl cyclase; OB, olfactory bulb; NO, nitric oxide; GC, nitric oxide sensitive guanylyl cyclase; NOsGC, nitric oxide sensitive guanylyl cyclase; nNOS, neuronal nitric oxide synthase; PI3K/PKB, PI3 kinase/protein kinase B; SFKs, Src family kinases; VLDLr, very low density lipoprotein receptor.

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doi:10.1016/j.jchemneu.2008.08.005

receptors and phosphorylation by Src family kinases (SFKs). Dab1 then activates the PI3 kinase/protein kinase B (PI3K/PKB) signalling cascade which targets numerous proteins affecting microtubuli and actin polymerization (Forster et al., 2006). ApoEr2 is more abundant than VLDLr in the forebrain and mediates Reelin signals in these brain regions (Jossin, 2004). Moreover, Reelin effects at maturing and adult synapses in the forebrain seem to be mediated by an ApoEr2 isoform which binds PSD-95 and the jun-kinase interacting proteins 1/2 (Beffert et al., 2005). Tethering of ApoEr2 to PSD-95 also targets it at NMDA receptors, in proximity of those SFKs (Src, Fyn) physically associated with such receptor. A reciprocal interaction between Dab1 and these SFKs occurs upon Reelin binding to the NMDA receptor complex, leading to its phosphorylation at tyrosine residues and eventually enhancing synaptic currents (Qiu et al., 2006).

NO signals support the synaptic changes underlying olfactory learning (Sanchez-Andrade et al., 2005) and nNOS and GC are abundant in interneurons of the olfactory bulb (OB) (Gutierrez-Mecinas et al., 2005; Herrmann et al., 2007; Hopkins et al., 1996; Kosaka and Kosaka, 2007). Reelin may, similarly to NO, also influence synaptic plasticity in the OB because reeler mutants exhibit deficits in olfactory learning (Larson et al., 2003). Moreover, expression of Reelin and nNOS in OB interneurons depends upon neurotransmission at olfactory receptor neuron–mitral cell synapses, since it is lost after sensory deafferentation (Okuyama-Yamamoto et al., 2005; Weruaga et al., 2000). Though differently regulated, NO and Reelin may thus accomplish related, partly complementary functions in the OB.

We recently showed that Reelin and nNOS are expressed in distinct interneuron subsets and that nNOS protein levels are low in the reeler OB. How could Reelin influence the content of nNOS? Maybe the ApoEr2 receptor links Reelin signalling to the calcium/calmodulin-mediated activation of nNOS via the NMDA receptor complex under control conditions and a deficit of extracellular Reelin results in an imbalance of such interplay (Herrmann et al., 2007). To sustain such hypothesis we tested in the present study whether ApoEr2 and nNOS are coexpressed in OB interneurons and defined the relative distribution of GC, Reelin and ApoEr2 in the OB. To essay how far changes in nNOS protein may indeed affect NO signalling, we compared the GC content of reeler and wildtype OB. Further, we analyzed if changes in nNOS and GC protein content occur in other reeler brain regions.

2. Materials and methods

2.1. Animals and tissue preparation

Adult 4–8 weeks old reeler Orleans mutants ($Reln^{rl\text{-}Orl}$) were used. Such mutation occurred spontaneously on a predominantly BALB/c background. The insertion of a L1 retrotransposon (ca. 7 kb) into the exon 61 of the Reeler gene by skipping the 220 bp-long exon leads to a truncated protein, which is not excreted (Takahara et al., 1996). Breeding pairs were kindly provided by A. Goffinet, Louvain University, Brussel, Belgium. Breeding was performed by mating viable and fertile homozygous animals. BALB/c mice were used as wildtype. Mice were kept on a 24 h day–night cycle with food and water ad libitum. All experiments were performed in accordance with the Swiss animal protection laws and institutional guidelines.

For immunocytochemistry, mice (5 $Reln^{rl\text{-}Orl}$ and 5 BALB/c) were deeply anaesthetized by intraperitoneal injection (100 μl/100 g body weight) of prequillan (0.5 mg/ml), xylapan (5 mg/ml) and narketan (50 mg/ml) and perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4.

For immunoblotting, animals (10 $Reln^{rl\text{-}Orl}$ and 10 BALB/c) were killed under deep fluothane anaesthesia. The brains were removed, different brain regions were rapidly dissected and frozen in liquid nitrogen and stored at −80° for 24–72 h prior further processing.

2.2. Immunocytochemistry and confocal image restoration

Vibratome sections, 40 μm thick, were processed for multiple immunofluorescent labelling as early described (Herrmann et al., 2007). We used the following primary antibodies: rabbit anti-NOS (aa.1409–1429, Sigma), mouse anti-Reelin 1:2000 (G10, Chemicon), mouse anti-ApoEr2 (clone 2H8) 1:1000 (kindly provided by A. Goffinet), rabbit anti-ApoEr2 (220) 1:1000 (kindly provided by J. Nimpf), rabbit anti-GCβ1 1:1000 (Cayman), rabbit anti-calretinin 1:5000 (7699/4, Swant), rabbit anti-calbindin D28K 1:5000 (CB 38 Swant), mouse anti-GAD 65 1:500 (GAD-6, DSHW, Iowa) and guinea pig anti-doublecortin 1:5000 (Chemicon). Primary antibodies were diluted in Tris buffered saline (5 mM, pH 7.6) with 5% normal goat serum (Vector Labs. Inc.) and incubated overnight at RT. Controls were performed omitting the first antibody. The rabbit and mouse ApoEr2 antibodies were tested by a double labelling protocol to ensure comparability between the two labelling patterns. In addition, they were tested in ApoEr2 ko mice (kindly provided by H.H. Bock, Freiburg, Germany). The secondary antibodies (Jackson Immunores., multiple labelling grade), either rhodamine (TRITC)-conjugated or fluorescein (FITC)-conjugated, were diluted 1:100 in phosphate buffered saline (13 mM, pH 7.4) and applied for 90 min at RT. Sections were coverslipped with 80% (v/v) glycerol and 0.2% paraphenyl diamine in 0.1 M phosphate buffer (pH 8.6). A Zeiss LSM 510 Meta equipped with an argon laser (488 nm) and a HeNe laser (543 nm) was used for image recording. Image processing was performed with the public domain Java based Image J software and consisted mainly of sigma filtering, γ adjustment and, in some cases, of maximum intensity *z* projections of 3–4 consecutive slices.

2.3. Immunoblotting

Homogenates of OBs, cortex/striatum and hippocampi were homogenised at 4 °C in PBS containing 1% Triton X-100 and 1 mM of the protease inhibitor phenylmethyl sulfonyl fluoride. After 10 min incubation of the lysate in this buffer, insoluble material was removed by centrifugation (Herrmann et al., 2007). The protein concentration of each sample was measured by the BCA Protein Assay (Pierce) and adjusted to 2 mg/ml in 60 mM Tris–HCl containing sodium dodecyl sulfate (3%, w/v), 1,4-dithiothreitol (2 mM), bromophenol blue (0,003%, w/v), glycerol 10% (v/v). Lanes were loaded with 50 μg protein. A prestained molecular weight dual color marker (Biorad) was loaded in an additional lane. Samples were run on a 7.5% w/v (for nNOS detection) or 10% w/v (for GCβ1 detection) polyacrylamide gel, and were then transferred to nitrocellulose membrane filters (Protran, Schleicher & Schuell). After a blocking step with 5% (w/v) fat-free dry milk powder in washing buffer (0.1%, v/v, Tween-20 in phosphate buffered saline), the blot matrices were incubated with rabbit anti-nNOS (1:5000) or with rabbit anti-GCβ1 (1:5000) overnight (4 °C) and subsequently for 90 min with a peroxidase-conjugated secondary antibody (1:5000, Biorad) at RT. Specific antigen–antibody complexes were detected by chemiluminescence using the Immun-Star™ HRP chemiluminescence kit (Biorad) and visualized by exposing the blot matrices to Kodak XR-5 films for 5 min.

Equal protein loading was confirmed on membranes by Ponceau S staining (Sigma). Since the band pattern and intensity was consistent for reeler and wildtype samples whenever the same protein amounts were loaded, selected immunoblots were used for semiquantitative densitometric analysis. The 160 kDa band corresponding to the nNOSα isoform and the 70 kDa band corresponding to the β1 subunit of GC were quantified on five separate blots using the Image J software.

3. Results

Fig. 1a–e illustrates the distribution of nNOS and ApoEr2 in the glomerular layer of the OB of Balb/c mice, the background strain of the $Reln^{rl\text{-}Orl}$ mice mutants. In combination with the rabbit anti-nNOS antibody we used the monoclonal antibody clone 2H8, for ApoEr2 detection (Jossin et al., 2004; Cariboni et al., 2005). nNOS labelled juxtaglomerular neurons, include periglomerular cells, tufted cells and superficial short axon neurons (Herrmann et al., 2007; Kosaka and Kosaka, 2007). External tufted cells and short axon neurons are difficult to unequivocally identify by their neurochemical characteristics, because there is no marker that labels them selectively (Parrish-Aungst et al., 2007). Since, however, there are specific markers for periglomerular cells (Kosaka and Kosaka, 2005, 2007), they may be recognized as putative external tufted and short axon cells per exclusion. Classical morphological cues like the size and position of the cell bodies and the orientation of their main dendrites also help to distinguish them from periglomerular cells (Kosaka and Kosaka, 2007; Pinching and Powell, 1971).

Neurons expressing ApoEr2 appear heterogeneous too. Numerous ApoEr2 immunoreactive large cell bodies distributed at the lower border of the glomerular layer resemble superficial short axon and external tufted cells by size and position and numerous appear to partially coexpress nNOS (Fig. 1a, b, d–d″, e–e″).