Accepted Manuscript

Title: Ndfip1 expression in developing neurons indicates a role for protein ubiquitination by Nedd4 E3 ligases during cortical development

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PII: S0304-3940(13)00833-1
DOI: http://dx.doi.org/doi:10.1016/j.neulet.2013.09.017
Reference: NSL 30049

To appear in: Neuroscience Letters

Received date: 2-4-2013
Revised date: 21-6-2013
Accepted date: 4-9-2013

Please cite this article as: C.-P. Goh, L.-H. Low, U. Putz, J. Gunnersen, V. Hammond, J. Howitt, S.-S. Tan, Ndfip1 expression in developing neurons indicates a role for protein ubiquitination by Nedd4 E3 ligases during cortical development, Neuroscience Letters (2013), http://dx.doi.org/10.1016/j.neulet.2013.09.017

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Highlights

- Ndfip1 protein expression was investigated in the developing neocortex during embryonic and postnatal stages
- Ndfip1 antibody specificity was confirmed using immunocytochemistry and Western blot analysis
- Ndfip1 and Nedd4-2 were co-extensively expressed in mitotic and post-mitotic neurons
- Ndfip1 was present in both pyramidal and non-pyramidal neurons
Ndfip1 expression in developing neurons indicates a role for protein ubiquitination by Nedd4 E3 ligases during cortical development.

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**ABSTRACT**

During development, protein substrates need to be removed and degraded when they are no longer required. The E3 ubiquitin ligases, including Nedd4 family proteins, are a major group of enzymes responsible for adding ubiquitin chains to protein substrates prior to their degradation. Ndfip1 (\textit{Nedd4 \_family\_interacting \_protein \_1}) is an adaptor and activator for Nedd4-family ubiquitin ligases for increasing substrate specificity. To study Nedd4-mediated ubiquitination during cortical development, we have mapped the spatio-temporal dynamics of Ndfip1 protein expression by immunocytochemistry. Ndfip1 expression was observed from embryonic day 11 (E11.5) until adult stages. Its presence increased during the postnatal stages and peaked at postnatal day 7 (P7). Spatially, Ndfip1 was found in the ventricular and marginal zones during corticogenesis but also in the cortical plate and subplate during midstage cortical development.
Postnatally, Ndfip1 was expressed in all cortical neurons (but not in glial cells) and this expression was both ubiquitous and uniform across cortical layers involving both pyramidal and non-pyramidal neurons. This consistent but dynamic pattern of Ndfip1 expression in temporal and spatial domains of the cortical landscape is indicative of complex programs of protein ubiquitination during corticogenesis.

Keywords: Ndfip1; Nedd4; cerebral cortex; protein expression; cortical development.

Abbreviations:

Ndfip1, Nedd4 family-interacting protein 1; Nedd4, Neural precursor cell-expressed developmentally down-regulated gene 4; Robo, roundabout; Comm, Commissureless; PTEN, Phosphatase and tensin homolog deleted chromosome 10.

1. Introduction

Ndfip1 is an endosomal transmembrane protein which contains two PPxY motifs that can bind to WW domains of Nedd4 ubiquitin ligases [10]. Ndfip1 can bind to protein targets that are normally unrecognized by Nedd4 E3 ligases for ubiquitination, increasing specificity and repertoire of substrates. In turn, Ndfip1 is capable of binding to multiple Nedd4 proteins, including Nedd4-1, Nedd4-2, ITCH and WWP2. Besides its role as an adaptor, Ndfip1 is also an activator of Nedd4 ligases by changing its passive to active conformation [12].
Although the function of Ndfip1 in the developing nervous system is unclear, there is ample evidence that its binding partner Nedd4 is implicated in neurodevelopment, including regulating axon guidance and dendrite development [2]. For example, *Drosophila* Nedd4 (dNedd4) inhibits the interaction of Robo (Roundabout) with Slit by the ubiquitination of Commissureless (Comm), a protein that controls Robo. This interaction is important for promoting midline axon crossing [13].

During corticogenesis, a number of cellular events are necessary to ensure coordinate implementation of neuron proliferation programs with neuron migration and differentiation. These events require rapid turnover and transit of proteins to effect rapid changes in cell states and cell behavior. Ubiquitin modification of proteins is therefore an important means of altering protein availability and abundance either by proteasome degradation or protein trafficking [5]. In the case of Ndfip1, our studies have shown that ubiquitination of its substrates can result in either substrate degradation [8] or trafficking [7, 15]. However, its function in the developing cortex is still unknown. We hypothesize that Ndfip1 is differentially expressed in morphogenetic regions of the developing cortex to effect selective ubiquitination of target proteins associated with cortical neuron generation and migration. This information will directly lead to future identification of ubiquitinated proteins accompanying these morphogenetic programs.

2. Materials and methods

2.1 Tissue preparation

C57BL/6J mice were obtained from Animal Resources Centre, Australia and bred to provide cortical samples for immunocytochemistry. The morning of the vaginal plug was defined as embryonic day E0.5. Embryos brains at E9.5 to E15.5 were dissected and fixed in 4%
paraformaldehyde in phosphate buffer (PB). Older embryos and pups were transcardially perfused with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The brains were post-fixed in 4% paraformaldehyde for 1 hr before equilibration with 20% sucrose in PB. The brains were embedded in OCT (Tissue-Tek, Torrance, CA) for cryosectioning. Coronal sections (12 μm) were obtained using a cryostat.

2.2 Antibody specificity

Mouse embryonic fibroblasts (MEFs) from Ndfip1 knockout (KO) mice [14] were grown to 80% confluence in 10 cm dishes in 15 ml medium (10% fetal calf serum, 50 U penicillin, 50 μg streptomycin, 4 mM L-glutamate in Dulbecco’s modified Eagle’s medium) (Invitrogen, Carlsbad, CA). Cells were transfected with a pcDNA3-Ndfip1-FLAG plasmid [16] using Effectene (Qiagen, Hilden, Germany) and immunocytochemistry was performed 24 hr later after fixation in 4% paraformaldehyde. Cells were incubated with a rat monoclonal anti-Ndfip1 (1:1000, generated in house) and mouse monoclonal anti-FLAG antibody (1:1000, Sigma) either alone or in combination. Secondary antibodies were: Alexa Fluor 594-conjugated goat anti-rat IgG (1:700; Molecular Probes) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500; Molecular Probes).

2.3 Western blotting analysis

To obtained whole cell lysates, Ndfip1 KO MEFs and wild-type MEFs were washed twice with ice-cold PBS and harvested with ice-cold RIPA buffer (50mM Tris, 100mM NaCl, 5mM EDTA, 1% TritonX-100, 0.2% SDS) and protease inhibitor cocktail (Roche Applied Sciences). For electrophoresis, 15 μg of protein was boiled at 95°C in Laemmli sample buffer for 5 min and electrophoretically separated on a 12% SDS-polyacrylamide gel, and blotted onto nitrocellulose.
membranes. Western blots were incubated overnight at 4°C in Ndfip1 monoclonal antibody (1:1000) in blocking solution. The blots were then incubated with anti-rat horseradish peroxidase antibody. Blots were visualized using Amersham ECL reagent (GE Healthcare).

2.4 Immunohistochemistry

Cryosections (12 μm) were rinsed with PB for 5 min followed by 0.3% TritonX-100 in PB for 5 min. The slides were incubated in blocking solution (10% fetal calf serum, 0.1% TritonX-100 in PB) for 1 hr at room temperature. The following primary antibodies were used: anti-Ndfip1 (1:500 rat monoclonal), anti-GABA (1:1000, Sigma, rabbit polyclonal), anti-Tbr1 (1:125, rabbit polyclonal), anti-Emx1 (1:500, rabbit polyclonal), anti-calretinin (1:200; Swant, Bellinzona, Switzerland, mouse polyclonal), anti-reelin (1:200; Millipore, Billerica, MA, mouse monoclonal), anti-pH3 (1:200; Millipore, Billerica, MA, rabbit polyclonal), anti-MAP2 (1:250; Sigma, St. Louis, MO, mouse monoclonal) and anti-Nedd4-2 (1:250; gift from Dr. Sharad Kumar, Adelaide, rabbit polyclonal). Secondary antibodies were: Alexa Fluor 594-conjugated goat anti-rat IgG (1:700; Molecular Probes), Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500; Molecular Probes) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500; Molecular Probes). Slides were incubated overnight at room temperature with primary antibodies followed by 1 hr in secondary antibodies before coverslipping. Images were captured using an Olympus Optical (Tokyo, Japan) microscope equipped with a Spot digital camera and ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Confocal images were collected on an inverted Axiovert 200-LSM5 Pascal confocal microscope (Zeiss, Oberkochen, Germany). The experiments were repeated on at least 3 different brains for each time point.
3. Results

3.1 Ndfip1 antibody specificity

A rat monoclonal antibody was raised against a peptide containing the first 116 amino acids of human Ndfip1. To test for antibody specificity, a FLAG-tagged Ndfip1 plasmid was expressed in Ndfip1-KO MEFs and immunostained. The results showed Ndfip1 immunoreactivity only in Ndfip1-FLAG expressing cells, but not in untransfected Ndfip1 KO MEFs (Fig. 1A-C). In Western blots, the Ndfip1 antibody recognized a correct-sized 30kDa band in the wild-type but not KO MEFs (Fig. 1D).

3.2 Ndfip1 in the embryonic cortex

Immunocytochemistry was conducted on coronal brain sections from E9.5 to P28. Ndfip1 expression was first observed at E11.5 in the ventricular zone (VZ) and the emerging preplate (PP) (Fig. 2A, 2B). Ndfip1 cells in the PP were positive for calretinin (Fig. 2C, inset), suggesting them to be Cajal-Retzius cells. Thus, Ndfip1 at E11.5 was expressed in both dividing (VZ) and non-dividing (PP) neuronal precursors. At E13.5, the PP is split into the marginal zone (MZ) and subplate (SP); both of these structures were stained for Ndfip1 (Fig. 2D). SP cells can be identified by their calretinin-immunoreactivity (Fig. 2E, insets). Postmitotic pyramidal neurons in the cortical plate (CP) can be identified by Tbr1 immunoreactivity (Fig. 2F, insets). Ndfip1 expression was most prominent on the pial aspects of the cortex, with positive cells detected in the MZ (Fig. 2G) where Reelin-positive cells are known to reside (Fig. 3H). Confocal microscopic views of boxed area (in Fig. 2H) revealed that some of the Reelin-positive cells were stained for Ndfip1 (Fig. 2I-K).
At E13.5, Ndfip1 expression in the VZ was reduced (Fig. 3A-B), but higher power views indicated that some of these were dividing, as revealed by the phospho-Histone H3 marker (Fig. 3C-E). By E15.5, Ndfip1 was barely detectable in the VZ (Fig. 3F). However, postmitotic neuroblasts in the CP were strongly stained, as were the subplate cells identified by calretinin (Fig. 3G, higher power views of boxed area). In the CP, future layer V and VI neurons with Ndfip1 could be identified by their immunoreactivity to Tbr1 (Fig. 3H, insets).

### 3.3 Ndfip1 in the postnatal cortex

At P1, Ndfip1 expression was widespread in the CP but less so in the germinal zones of the VZ (Fig. 3I). Within the CP, Ndfip1 staining was polarized, with greater staining intensity for neurons lying in the upper but not lower layers of the CP (Fig. 3I). Conversely, Tbr1-positive neurons which mark lower layer neurons had lower levels of Ndfip1 (Fig. 3J, K and insets) creating a complementary pattern against Ndfip1 (Fig. 3K). At P7, Ndfip1-staining of cortical neurons was widespread, and higher power confocal views showed the protein to be present in the cell soma, and also in the apical dendrite revealed by the dendritic marker, MAP2 (Figs 4A-C). By P28, all projection neurons labeled for Tbr-1 were also Ndfip1-positive (Figs. 4D-F; and Figs. 4G-I, higher power views). We confirmed that Ndfip1 was exclusively expressed in neurons and not glia [18]. We surmised that the pattern of Ndfip1 expression in the postnatal cortex was a reflection of neuron density and arrangement, with greater staining intensity in the larger sized layer V pyramidal neurons, and correspondingly lesser intensity in the smaller sized layers II/III pyramidal neurons (Fig. 4D).
3.4 Ndfip1 in pyramidal and non-pyramidal neurons

The cortex is constituted by a majority of pyramidal neurons (80 percent) and non-pyramidal interneurons (20 percent). Although sharing a common cortical space, these two neuronal populations have different origins, with the pyramidal neurons arising locally from the cortical neuroepithelium [17], but the interneurons arising from the subcortical ganglionic eminence from where they undertake long-distance tangential migration [1, 20]. The results indicated that Ndfip1 was expressed by both pyramidal neurons (marked by Emx1) (Fig. 5A-C) and the non-pyramidal interneurons (marked by GABA) (Fig. 5D-F). Together all Ndfip1-positive cells were accounted for by staining for either Emx1 or GABA suggesting that all neurons, but not glia, possessed Ndfip1 (Fig. 5).

3.5 Co-localization of Ndfip1 and Nedd4-2 in developing neurons

Ndfip1 is capable of binding to both Nedd4-1 and Nedd4-2 [19], suggesting that the choice of E3 ligase for ubiquitinating protein targets was critical. Nedd4-1 is important for dendritic development but is known to disappear by P21 [11], therefore we focused on Nedd4-2 expression paying particular attention to its spatio-temporal regulation with respect to Ndfip1. The results indicated remarkable similarity, both in the timing and the specificity of staining patterns (Fig. 6). At E13.5, both proteins exhibited strong staining in the upper parts of the cortical wall and co-extensively present in the same neurons (Fig. 6A-C). A similar picture emerged at E15.5, where Ndfip1 staining in cortical plate neurons was co-extensive with Nedd4-2 (Fig. 6D-F).
4. Discussion

Proper development of the brain requires the timely expression of proteins to drive a multitude of morphogenetic events such as neuron division, migration, polarity and differentiation. Following initial positioning of neurons at their correct addresses, appropriate development of dendritic arbors and excitatory synaptic connections is crucial for cortical wiring. To drive these developmental events, new proteins need to be expressed whereas old proteins that are no longer required have to be removed. Failure of molecular control in protein expression or distribution is a certain recipe for migration perturbations causing neurodevelopmental disorders [3] [4].

A central mechanism for protein removal and turnover is by ubiquitination, and a number of studies attribute Nedd4 ubiquitin ligases with crucial roles in neurite outgrowth [2]. Nedd4-1 is implicated in enhancing dendritic branching in mouse neurons [11]. However, there is no information on the developmental profile of Ndfip1, an important adaptor and activator for Nedd4 ligases [12].

The present report therefore establishes new ground for studying ubiquitination by Nedd4 E3 ligases during cortical morphogenesis. This is because Ndfip1 can recognise substrates beyond the normal targets recognized by Nedd4. In the embryonic cortex, Ndfip1 was present during the most active phases of cell division in the germinal zones, and cell migration into the PP. Ndfip1 was expressed at these critical stages (E11.5 to E13.5) in both mitotic and post-mitotic neurons. At mid-corticogenesis stage (E15.5), post-mitotic neurons that accumulate in the CP are known to be undergoing maturation by extending cortico-thalamic and cortico-cortico efferents, while at the same time receiving thalamo-cortical afferents. Their cell bodies, marked by Tbr-1, were strongly positive for Ndfip1 and ubiquitin ligase Nedd4-2, suggesting the ubiquitination of yet-
to-be identified protein substrates. Interestingly, Ndfip1 expression at post-natal (P1, P7) and juvenile (P28) stages was strongly upregulated in all cortical neurons, whether they be glutamatergic or GABAergic. From our previous studies, we know that Ndfip1 function in adult neurons, whether excitatory or inhibitory, is important for improving neuron survival following brain injury and cerebral ischemia [6, 18].

In conclusion, the expression profile of Ndfip1 during different developmental stages, and its ubiquitin ligase Nedd4-2 during midcorticogenesis, suggests that ubiquitin-mediated alterations in protein levels and protein turnover are required for neuron migration, positioning and maturation. While there are a number of known substrates for Ndfip1, such as transcription factor Jun B [14], divalent metal transporter 1 [9], tumor suppressor PTEN [6], it is likely that the future studies will increase this tally.

**Figure legends**

Fig. 1. Ndfip1 antibody is specific. (A-C) Ndfip1-KO MEFS transfected with FLAG-tagged Ndfip1 shows Flag and Ndfip1 staining (arrow heads). Ndfip1 is not present in untransfected Ndfip1-KO cells (arrows). (D) Western blot analysis of protein lysates from wild-type and KO MEFs demonstrates antibody specificity for Ndfip1. Scale bar: A-C: 10 μm

Fig. 2. Ndfip1 expression during early cortical development. (A) Ndfip1 immunoreactivity (red) in a coronal section of the E11.5 forebrain. (B) Higher magnification shows that Ndfip1 is present in the PP and VZ. Inset shows Ndfip1 staining in PP cells. (C) Calretinin-positive cells in the PP expressed Ndfip1. Inset shows double-staining for both proteins in PP cells. (D) Ndfip1 expression at E13.5 in the MZ and VZ. (E) Ndfip1-positive cells in the emerging cortical plate
also contain calretinin (arrows in insets indicate double-staining). (F) Ndfip1 is present in Tbr1-positive cells of the upper cortical plate (arrows in insets indicate double-staining). (G, H) Ndfip1 in the MZ is frequently found within Reelin-positive cells. (I-K) Confocal views of boxed area confirm double staining for Ndfip1 and Reelin. Scale bar: A: 250 μm; B-C: 25 μm; D: 250 μm; E: 25 μm; F: 20 μm; G-H: 100 μm; I-K: 10 μm

Fig. 3. Ndfip1 is present during midcorticogenesis and early postnatal stage. (A-E) Ndfip1 is present inside dividing cells of the E13.5 VZ (arrows), evidenced by double staining for the mitotic marker, phospho-histone pH3. Higher power views of boxed areas show strong co-expression of Ndfip1 (arrows). (F-H) At E15.5, Ndfip1 staining is strong in the SP, marked by calretinin immunoreactivity (arrows in insets), and also in pyramidal neurons expressing Tbr1 (arrows in insets). (I-K) At P1, Ndfip1 staining can be seen throughout the cortex, with stronger signals present within neurons of the mid upper layers although Tbr-1 positive neurons in the lower layers are also strongly stained for Ndfip1 (insets of boxed areas). Scale bar: A-B: 100 μm; C-E: 15 μm; F: 300 μm; G-H: 100 μm; I-K: 150 μm

Fig. 4. Ndfip1 expression in postnatal and juvenile neocortex. (A-C) Confocal views of P7 neocortex shows Ndfip1 is expressed in dendrites of pyramidal neurons stained with MAP2 (arrows). (D-I) Ndfip1 is expressed in all pyramidal neurons positive for Tbr-1. Higher power views of boxed areas show strong colocalization of both proteins. Scale bar: A-C: 10 μm; D-F: 60 μm; G-I: 30 μm

Fig. 5. Ndfip1 is expressed in both pyramidal and non-pyramidal neurons. (A-C) Ndfip1 is expressed in all pyramidal neurons marked by the Emx1 marker (arrows). (D-F) Ndfip1 is present in GABAergic interneurons (arrows). Scale bar: A-C: 30 μm; D-F: 20 μm
Fig. 6. Nedd4-2 is expressed in Ndfip1-positive cells in the developing neocortex. (A-C) In the E13.5 cortex, both Ndfip1 and Nedd4-2 are strongly expressed in the upper part of the cortical wall, including the MZ. Higher magnification of the boxed area shows colocalization of both proteins (insets). (D-F) At E15.5, both Ndfip1 and Nedd4-2 are expressed in the CP and MZ, with colocalization of both proteins in certain cells (insets). Scale bar: A-C: 50 μm; D-F: 80 μm

Acknowledgements:

This work was supported by the National Health and Medical Research Council (NHMRC) and the Victorian Government through the Operational Infrastructure Scheme. We thank Dr. Sharad Kumar the Nedd4-2 antibody.

References:


Figure 2

E11.5

A

PP

LV

VZ

Ndflp1

B

PP

VZ

Ndflp1

C

Ndflp1

Calretinin

E13.5

D

MZ

VZ

LV

LGE

Ndflp1

E

Ndflp1

Calretinin

F

Ndflp1

Tbr1

G

MZ

VZ

LV

Ndflp1

H

Ndflp1

Reelin

I

MZ

Ndflp1

J

Reelin

K

Overlay
Figure 4

(A) Ndfip1 expression at P7.
(B) MAP2 staining at P7.
(C) Overlay of Ndfip1 and MAP2.
(D-I) Layer distribution of Ndfip1 at P28.
(J-L) Enlarged view of Ndfip1 and Tbr1 expression in layers VI and I.
Author/s:
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Title:
Ndfip1 expression in developing neurons indicates a role for protein ubiquitination by Nedd4 E3 ligases during cortical development

Date:
2013-10-25

Citation:

Persistent Link:
http://hdl.handle.net/11343/44162