



## Comparative distribution of protein components of the A20 ubiquitin-editing complex in normal human brain

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### HIGHLIGHTS

- Components of the A20 ubiquitin-editing complex are present in the human brain.
- A20 complex components are mainly expressed in neurons.
- A20 complex components are differentially expressed in various regions of the human brain.

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### ABSTRACT

Activation of innate and adaptive immune responses is tightly regulated, as insufficient activation could result in defective clearance of pathogens, while excessive activation might lead to lethal systemic inflammation or autoimmunity. A20 functions as a negative regulator of innate and adaptive immunity by inhibiting NF-κB activation. A20 mediates its inhibitory function in a complex with other proteins including RNF11 and Itch, both E3 ubiquitin ligases and TAX1BP1, an adaptor protein. Since NF-κB has been strongly implicated in various neuronal functions, we predict that its inhibitor, the A20 complex, is also present in the nervous system. In efforts to better understand the role of A20 complex and NF-κB signaling pathway, we determined regional distribution of A20 mRNA as well as protein expression levels and distribution of RNF11, TAX1BP1 and Itch, in different brain regions. The distribution of TRAF6 was also investigated since TRAF6, also an E3 ligase, has an important role in NF-κB signaling pathway. Our investigations, for the first time, describe and demonstrate that the essential components of the A20 ubiquitin-editing complex are present and mainly expressed in neurons. The A20 complex components are also differentially expressed throughout the human brain. This study provides useful information about region specific expression of the A20 complex components that will be invaluable while determining the role of NF-κB signaling pathway in neuronal development and degeneration.

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**Abbreviations:** CNS, central nervous system; GUSB, glucuronidase-beta; HEK293, human embryonic kidney 293; qRT-PCR, quantitative real-time PCR; RNF11, RING finger protein 11; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Tax1, human T-cell leukemia virus type I; TAX1BP1, Tax1 binding protein 1; TNFAIP3, tumor necrosis factor, alpha-induced protein 3; TRAF, tumor necrosis factor receptor-associated factor.

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### 1. Introduction

The NF-κB signaling pathway is well known for its ubiquitous roles in inflammation, immune responses, and control of cell division and apoptosis [21]. These roles of NF-κB signaling are apparent in the central nervous system (CNS) where they can range from neuronal development, synaptic signaling that underlies learning and memory and coordination of immune responses to toxic stimuli [11,14]. Activation of NF-κB is normally transient, and persistent

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NF- $\kappa$ B activation is associated with several autoimmune diseases and cancer in the peripheral system [25] and in both acute (i.e., stroke, seizures) and progressive (Parkinson's disease, Alzheimer's disease, Amyotrophic Lateral Sclerosis) neurodegenerative disease [12]. However, inhibition of NF- $\kappa$ B signaling can prevent adult neurogenesis in the dentate gyrus [14]. Dysregulation of NF- $\kappa$ B signaling has been cited as a potential source of synaptic pathology in AD and PD due to the importance of NF- $\kappa$ B transcriptional regulation of spine density in mature neurons [6]. Thus, duration of NF- $\kappa$ B activation is tightly regulated causing genes that play key roles in amplification and effector functions to be actively repressed under basal conditions [28]. One such regulator of NF- $\kappa$ B activation is A20 (also known as TNFAIP3), an ubiquitin-editing protein, which regulates NF- $\kappa$ B activation in a negative feedback loop [28]. Specifically, A20 contains several NF- $\kappa$ B binding sites within the promoter of the human gene to induce its expression in response to NF- $\kappa$ B signaling [16]. Recent experiments have established that A20 mediates its inhibitory function in a complex with three other proteins, Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1, also called TXBP151 or T6BP), and two E3 ubiquitin ligases, Itch (also known as AIP3) and RING finger protein 11 (RNF11) [23,24]. The inhibitory role of A20 is critically dependent on its interaction with other complex members, TAX1BP1, Itch and RNF11. TRAF6, a member of the tumor necrosis factor receptor-associated factor (TRAF) family, and a known substrate of A20 is also an E3 ligase [23,24]. To better understand the role of A20 complex in the CNS we determined the presence and distribution of RNF11, TAX1BP1, Itch and TRAF6 proteins in normal human brain. Since A20 is an inducible protein [5], we have determined A20 mRNA expression levels in various normal human brain regions.

## 2. Materials and methods

### 2.1. Cell culture

Human embryonic kidney 293 (HEK293) were purchased from American Type Culture Collection and were maintained at 37 °C and 5% CO<sub>2</sub>. HEK293 cells were cultured in DMEM with 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (Bio Whittaker).

### 2.2. Human tissue

Post-mortem, control (pathologically clean) brains ( $n=3$ ) were obtained through Emory University's Brain Bank. The average age at death of the samples was 64 years. Blocks of formalin fixed tissue at the level of cerebral cortex, midbrain, pons, or inferior olive were sectioned on a freezing microtome. Free-floating sections (50  $\mu$ m) were stored at –20 °C in ethylene glycol cryopreservative.

### 2.3. Immunohistochemistry

Immunohistochemistry for single-label light microscopy and double-label fluorescence microscopy has been described previously [4]. For light microscopy, 3,3'-diaminobenzidine was used as chromophore. Routine hematoxylin was used to counter-stain nuclei in hippocampal sections following single labeling. For fluorescent microscopy, appropriate secondary antibodies with fluorescent chromophores were used. For control experiments, primary antibodies were omitted. Immunostained sections were imaged using bright-field Olympus microscope or using a Zeiss LSM 510 laser scanning confocal microscope. Images were processed using Adobe Photoshop 7.0 software.

### 2.4. Antibodies

Antibodies used were: A20 (ab13597, Abcam), A20 (NBP1-40684, Novus), A20 (550859, BD Pharmingen), A20 (sc-166692, Santa Cruz), GFAP (MAB360, Millipore), Iba1 (ab5076, Abcam), Itch (611198, BD Transduction), Olig2 (MABN50, Millipore), rabbit polyclonal RNF11 (described in [1]), TAX1BP1 (ab22049, Abcam), and Traf6 (ab13853, Abcam).

### 2.5. Plasmids and transfections

Flag-A20, Flag-Itch, Flag-TAX1BP1, and Flag-TRAF6 were a kind gift from Dr. Edward Harhaj (University of Miami). Transient transfections of HEK293 cells were performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol.

### 2.6. Quantitative real-time PCR

Total RNA was isolated from approximately 100 mg of human brain tissue that was homogenized and sonicated before using a RNeasy extraction kit (Qiagen). cDNA was created using a High Capacity cDNA reverse transcription kit (Ambion). Real-time PCR was performed on a 7500 Fast RT-PCR instrument (Applied Biosystems) using TaqMan PCR master mix (Applied Biosystems) and gene-specific TaqMan probes (Applied Biosystems) against RNF11 (Hs00702517.s1), A20 (Hs00234712.m1) and glucuronidase-beta (GUSB) (Hs99999908.m1). RNA samples were run in triplicate. Gene expression was normalized to the house-keeping gene GUSB and relative expression was calculated for each gene using  $2^{-\Delta\Delta C_t}$  method.

### 2.7. Immunoblotting

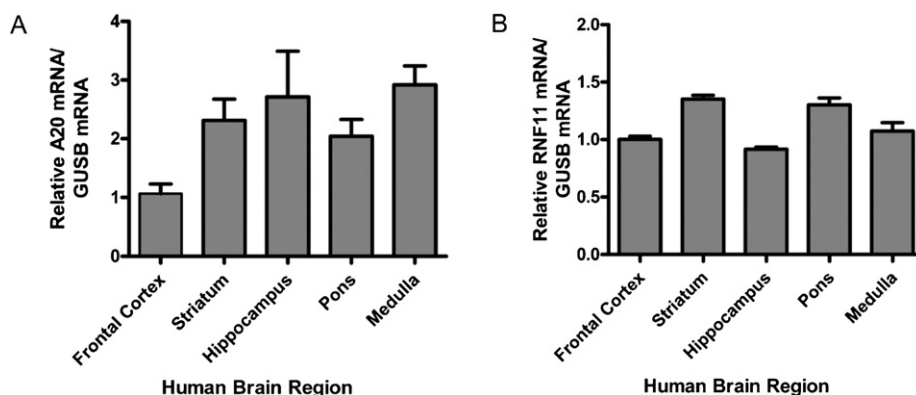
Immunoblotting was performed as previously described [1]. Cells were transfected for 24 h with indicated plasmids and harvested in PBS with 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.1% Triton 100 $\times$ , 1% NP-40, Halt phosphatase inhibitor cocktail (Pierce), and protease inhibitor cocktail (Roche Diagnostics). Samples were spun at 14,000 rpm before being separated by SDS-PAGE and transferred onto PVDF membranes. Membranes were scanned using the Odyssey Image Station (LiCor).

## 3. Results

Sections of adult normal brain tissue were immunostained following characterization of commercial antibodies, against RNF11, TAX1BP1, Itch and TRAF6. The regional distribution and expression levels of different proteins and A20 mRNA are described below.

### 3.1. Antibody characterization

To determine specificity of the antibodies used for immunohistochemistry, we looked at endogenous expression of complex members and overexpression of FLAG-tagged constructs in HEK293 cells (Supplemental Fig. S1). We tried numerous commercially available antibodies against A20 but they either did not label human brain tissue or the specificity could be confirmed by pre-adsorption. However, in HEK293 cells, endogenous levels of A20 were detectable at the predicted molecular weight for this protein using immunoblotting (Supplemental Fig. S1A). Endogenous expression levels of Itch, TAX1BP1, and Traf6 were also detectable with a band near their predicted molecular weight (Supplemental Fig. S1B–E). With confirmation that our antibodies were specific for the different A20 complex members, we set out to explore the distribution of the complex in different regions of the human brain.



**Fig. 1.** A20 is differentially expressed throughout human brain. (A) and (B). RNA from control human cases was analyzed by qRT-PCR for A20 and RNF11 expression, relative to GUSB. A20 and RNF11 are detectable in all brain regions examined.

### 3.2. A20 mRNA expression levels

Distribution of A20 mRNA in various brain regions from control cases was analyzed by quantitative real-time PCR (qRT-PCR). A20 mRNA was differentially expressed in various brain regions examined (Fig. 1A). In comparison to frontal cortex, A20 expression was increased in striatum, hippocampus, pons, and medulla (2.3, 2.7, 2.0, and 2.9 fold, respectively). Unlike A20, large differences in mRNA expression of RNF11 were not detected (Fig. 1B). The detection of A20 mRNA expression in the different regions suggested that an A20 ubiquitin-editing complex exists in these regions and thus, expression of the individual complex members was studied in human tissue.

### 3.3. Distribution of A20 complex components in cerebral cortex

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was abundantly detected in cerebral cortex of human brain (Fig. 2A and B). For this study we chose frontal and cingulate cortices for evaluation since they have the characteristic six cortical layers. Expression levels for all four proteins were detected in neurons in all the six isocortical layers. In particular, cell bodies of neurons in layers III, IV and V had the densest immunoreactivity. Small and large pyramidal and multipolar neurons and their processes were immunolabeled for all proteins. Furthermore, while immunoreactivity for RNF11 and Itch was detected mainly in neuronal cytoplasm, TAX1BP1 and TRAF6 immunoreactivity was detected in both neuronal cytoplasm and nuclei (Fig. 2A). Immunoreactivity for all proteins was observed in glia-like cells in white matter underlying the cortical tissue (Fig. 2B) as evident from location and cell morphology. Double-labeling with Iba1 confirmed microglial expression (Supplemental Fig. S3). Minimal colocalization was observed with GFAP (astrocytes) or Olig2 (oligodendrocytes) antibodies and A20 complex components (data not shown).

### 3.4. Distribution of A20 complex components in hippocampus

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in various regions of the hippocampal formation, consisting of dentate gyrus, hippocampus, subiculum, and entorhinal cortex. Immunoreactivity for all proteins was observed specifically in the homogenous pyramidal cell layer of CA1–3 fields of hippocampus (Fig. 2C) and granule cell layer of dentate gyrus. Similar to cortical tissue, immunoreactivity for all proteins was mainly detected in neuronal cells.

### 3.5. Distribution of A20 complex components in striatum

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in striatal medium-sized (mainly medium spiny neurons) and their processes (Fig. 2D). Additionally, immunoreactivity was observed in striatal large neurons (cholinergic neurons) and their processes. Limited immunoreactivity for the different proteins was detected in the fiber bundles crossing the striatum.

### 3.6. Distribution of A20 complex components in substantia nigra

Immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in large neurons of substantia nigra pars compacta, which have characteristic neuromelanin pigment (Fig. 2E). Immunoreactivity was observed in the smaller neurons of substantia nigra pars reticulata, which are typically GABAergic cells.

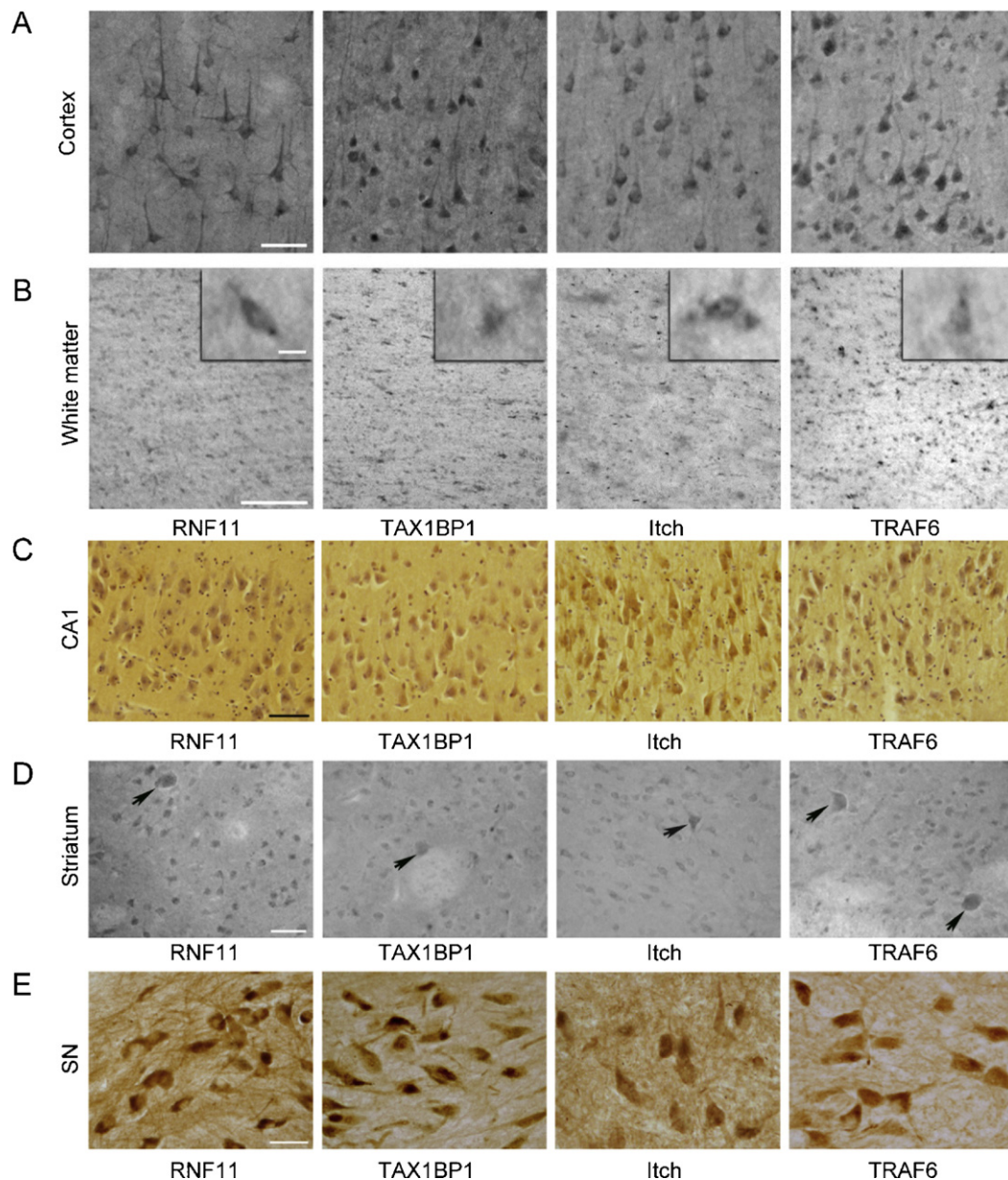
### 3.7. Distribution of A20 complex components in medulla

Immunoreactivity for RNF11, TAX1BP1, TRAF6 and Itch was detected in various brainstem regions (see Fig. 3A). Similar to other regions, immunoreactivity for all proteins was mainly detected in neurons. Neurons of the dorsal motor nucleus of vagal nerve and reticular formation were immunoreactive for all proteins (Fig. 3B). Similarly, neuronal immunoreactivity for RNF11, TAX1BP1, Itch and TRAF6 was detected in the convoluted bands of cells in the inferior olivary nucleus, positioned dorsal to the pyramids. Immunoreactivity for all proteins was detected in glial-like cells of the pyramids.

## 4. Discussion

In this study we determined the expression and distribution of A20 ubiquitin-editing complex components including A20, RNF11, TAX1BP1, Itch and TRAF6 in normal human brain in order to demonstrate the existence of this crucial regulator of NF- $\kappa$ B signaling pathway in CNS. Prior to immunohistochemical analysis, we examined specificity of commercial antibodies available for proteins in the A20 complex. In absence of peptides to perform pre-absorption experiments, we induced over-expression of proteins of interest in HEK293 cells and demonstrated that our antibodies were able to detect endogenous and over-expressed proteins. A similar protocol has been used to determine the specificity of commercial antibodies in past studies [18].

Our investigation demonstrated presence of TAX1BP1, Itch, RNF11 and TRAF6 in normal human brain. Furthermore, we showed that TAX1BP1, Itch, RNF11 and TRAF6 are differentially expressed in all brain regions examined, are mainly localized in neurons, and follow region-specific neuronal cytoarchitecture. A20 is an



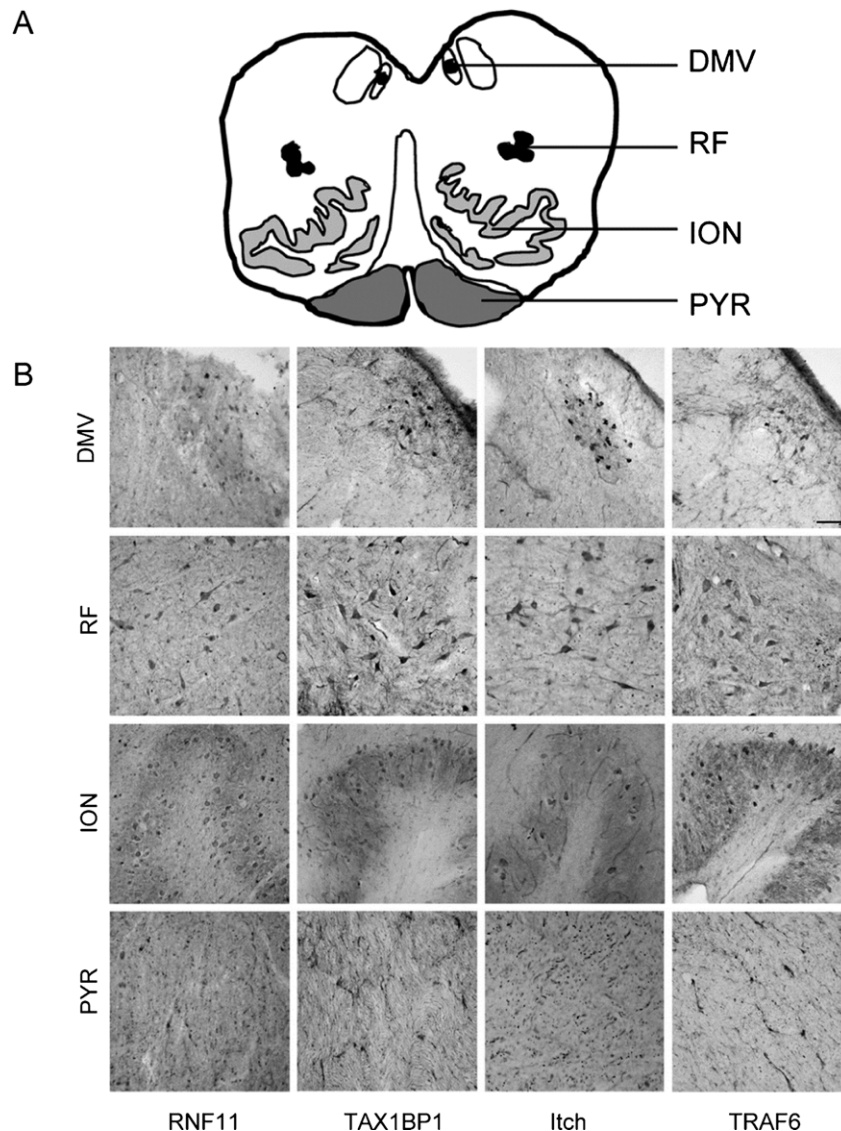
**Fig. 2.** Expression of A20 complex components in normal human brain. (A) RNF11, TAX1BP1, Itch and TRAF6 have abundant neuronal expression throughout cerebral cortex. RNF11 and Itch immunoreactivity (IR) was detected mainly in the cytoplasm while TAX1BP1 and TRAF6 IR was detected in both cytoplasm and nucleus. (B) In white matter underlying the cortex, IR for all proteins was detected in glia-like cells (inset shows magnification of individual cells). (C) IR for all proteins (brown precipitate) was detected in all pyramidal cells of CA1 in hippocampus (hematoxylin counterstain for nuclei shown in purple). (D) IR for all proteins was detected in the striatal medium-sized spiny neurons and large neurons (arrowhead). Limited IR for different proteins was detected in the fiber bundles of the striatum. (E) IR for all proteins (brown precipitate) was detected in large substantia nigra (SN) neurons with neuromelanin pigment (dense dark brown granules). Scale bar–50  $\mu$ m for all except 2B–100  $\mu$ m, inset–10  $\mu$ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

inducible, cytoplasmic protein, which is constitutively expressed in thymocytes and peripheral T cells with minimal expression elsewhere at steady state [26,29]. We were unable to detect specific A20 protein expression in the brain despite using four different commercially available antibodies. This prompted us to demonstrate the presence of A20 mRNA in various brain regions using qRT-PCR analysis of homogenized brain tissue containing both neuronal and glial cell populations. Our studies suggest that A20 protein has minimal expression in normal brain with the potential to be induced in response to appropriate stimuli, perhaps as observed following MPTP-treatment [19] or TNF stimulation in mice [17].

RNF11 is a putative E3 ubiquitin ligase with a RING-H2 domain. In humans, RNF11 expression is up-regulated in various cancers [7]. Additionally, RNF11 is a candidate gene for late onset

Parkinson's disease at the PARK10 locus [13] and colocalizes with  $\alpha$ -synuclein-positive Lewy bodies [1]. There are no other reports of RNF11 expression in the CNS and the Protein Atlas has yet to complete its analysis of RNF11 protein expression [26].

TAX1BP1 is an anti-apoptotic protein that is constitutively expressed in human cells and binds to TAX protein of the human T-cell leukemia virus type 1. TAX protein is localized in the nucleus and cytoplasm of infected cells. There it interacts with different host proteins such as TAX1BP1 in order to regulate viral and cellular transcription, signal transduction pathways, and apoptosis [27]. This would explain the cytoplasmic and nuclear localization of TAX1BP1 in neurons (Fig. 3). However there is no report as yet of TAX1BP1 expression and distribution in CNS, though the Protein



**Fig. 3.** Expression of A20 complex components in normal human brainstem region. (A) Schematic of the human medulla to illustrate various nuclei examined. (B) Neurons of the dorsal motor nucleus of vagal nerve (DMV) and reticular formation (RF) were immunoreactive for RNF11, TAX1BP1, Itch and TRAF6. Similarly, neuronal immunoreactivity for all proteins was detected in the convoluted bands of cells in the inferior olivary nucleus (ION). Immunoreactivity for RNF11, TAX1BP1, TRAF6 and Itch was detected in glia in the pyramids (PYR). Scale bar-100  $\mu$ m.

Atlas does confirm the cytoplasmic and nuclear localization of TAX1BP1 in neurons and cells [26].

Itch, a member of the NEDD4 family of E3 ubiquitin ligases, has four WW domains for specific interactions with PY motif-containing substrates. Itch knockout mice, similar to A20 and TAX1BP1 knockout mice, display severe immunological disorders associated in every organ in the body [3,20]. In humans, Itch is strongly expressed in the gastrointestinal tract, pancreas, neuronal cells and lymphoid tissues [20]. According to the Protein Atlas, Itch immunoreactivity is mainly detected in cytoplasm of cells [26], similar to our observations (Fig. 3).

TRAF6 is a member of the TRAF family of proteins that are involved in regulating cell death, survival and cellular responses to stress [2,9,11]. TRAF6 has been implicated in Parkinson's disease and traumatic brain injury [8,30]. As demonstrated in our studies, TRAF6 expression levels were detected in human, nigral dopaminergic neurons [30] while its cytoplasmic and nuclear localization has been identified in other human brain regions [26]. Neuronal

TRAF6 expression has also been reported in normal, adult rat brain [8].

As mentioned above, components of the A20 complex are expressed predominantly in lymphoid tissues that are actively involved in inflammatory responses outside of CNS. However, RNF11, TAX1BP1, Itch and TRAF6 are constitutively and highly expressed in neurons as compared to glial cells, suggesting that NF- $\kappa$ B activation is tightly regulated in neurons, the post-mitotic cells of the CNS, as an inherent protective strategy. Indeed, Kaltschmidt et al. found increased NF- $\kappa$ B activation in cells around plaques in normal and AD patients [15]. However, they also observed a down-regulation in NF- $\kappa$ B activity between early and late plaque stages, which was suggested to make these cells more sensitive to insults and degeneration. We do see expression of A20 complex components in microglial cells, the immune cells of the CNS, possibly due to their important role in inflammatory responses and initiation of NF- $\kappa$ B signaling pathways [10,22].

## 5. Conclusions

Our investigations, for the first time, describe and demonstrate that the essential components of the A20 ubiquitin-editing complex are present and mainly expressed in neurons. This study provides useful information about region specific expression of the A20 complex components that will be invaluable while determining the role of NF- $\kappa$ B signaling pathway, in normal and diseased brain. This will be specially important since signaling molecules that are involved in the activation and regulation of NF- $\kappa$ B-dependent gene expression have drawn much interest as potential targets for treatment of several autoimmune and neurodegenerative diseases.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.neulet.2012.05.043>.

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