The Neuronal and Peripheral Expressed Membrane-Bound UNC93A Respond to Nutrient Availability in Mice

Mikaela M. Ceder*, Emilia Lekholm, Sofie V. Hellsten, Emelie Perland and Robert Fredriksson

Molecular Neuropharmacology, Department of Pharmaceutical Biosciences, Uppsala University, Uppsala, Sweden

Many transporters such as the solute carriers belonging to the Major facilitator superfamily Pfam clan are orphans in that their tissue and cellular localization as well as substrate profile and function are still unknown. Here we have characterized the putative solute carrier UNC93A. We aimed to investigate the expression profile on both protein and mRNA level of UNC93A in mouse since it has not been clarified. UNC93A staining was found in cortex, hippocampus and cerebellum. It was found to be expressed in many neurons, but not all, with staining located in close proximity to the plasma membrane. Furthermore, we aimed to extend the starvation data available for Unc93a in hypothalamic cell cultures from mouse. We investigated the Unc93a alterations with focus on amino acid deprivation in embryonic cortex cells from mice as well as 24 h starvation in adult male mice and compared it to recently studied putative and known solute carriers. Unc93a expression was found both in the brain and peripheral organs, in low to moderate levels in the adult mice and was affected by amino acid deprivation in embryonic cortex cultures and starvation in vivo samples. In conclusion, the membrane-bound UNC93A is expressed in both the brain and peripheral tissues and responds to nutrient availability in mice.

Keywords: UNC93A, SLC, MFS, MFSD, transporter protein, starvation

INTRODUCTION

Membrane proteins make up one third of all proteins (Almen et al., 2009) and are of critical importance to nearly every aspect of cell physiology. Transporters are one group of membrane proteins that allows movement of molecules over lipid barriers making it possible for absorption, distribution, metabolism, and excretion (Hediger et al., 2013; Yan, 2015). Transporters of the solute carrier (SLC) family are one group of proteins underrepresented in research (Cesar-Razquin et al., 2015) and numerous transporters remain orphans where details about cellular location and/or function are missing. Traditionally transporters are classified into families based on structure and/or function (Schlessinger et al., 2013; Perland and Fredriksson, 2017) and the largest transporter family in humans is the SLC, comprising approximately 450 members currently divided into 52 subfamilies (Hediger et al., 2013; Cesar-Razquin et al., 2015; Perland and Fredriksson, 2017).
Recently, protein sequence analysis (Perland and Fredriksson, 2017; Perland et al., 2017a) found that the orphan membrane-bound protein UNC93A is closely related to SLCs belonging to the Major Facilitator superfamily (MFS) Pfam clan (CL0015), thereby classifying it as a putative SLC (Perland and Fredriksson, 2017). MFS is a large group of facilitators and secondary active transporters with a diverse substrate profile, function and expression patterns (Pao et al., 1998; Reddy et al., 2012; Yan, 2015). MFS members are significant for vital cellular physiology in both health and disease and it is therefore important to understand their role. The MFS members are present in several biological kingdoms (Hoglund et al., 2011), and UNC93A is conserved in insects, nematodes, rodents, and humans (Levin and Horvitz, 1992; Campbell et al., 2011; Son et al., 2015; Perland et al., 2017a).

Unc-93 was first identified in a genome-wide screening in Caenorhabditis elegans, in which it is suggested to be a potassium channel regulatory protein, one of five genes identified to be involved in muscle coordination (Levin and Horvitz, 1992; de la Cruz et al., 2003). In mammals, there are two UNC93 proteins, UNC93A and UNC93B1 (Perland and Fredriksson, 2017). So far there is little research on expression profile, localization and function of UNC93A, while UNC93B1 is reported to be a toll-like receptors (TLRs) signaling regulator, important for the trafficking of TLRs from the endoplasmic reticulum to endolysosomes (Lee et al., 2013). UNC93B1 is also important for TLR5 expression and function at the plasma membrane (Huh et al., 2014). There have been attempts to study the role of UNC93A in humans. Liu et al. (2002) studied an allele loss on chromosome 6 linked to sporadic ovarian cancers that indicated the presence of a putative tumor suppressor gene, where UNC93A was identified within the interval of the allele loss. They continued to evaluate UNC93A as the potential tumor suppressor gene, but Liu et al. (2002) concluded that UNC93A was plasma membrane bound but was not the tumor suppressor gene they searched for. UNC93A was also mentioned in a family based association study, as a candidate for pulmonary function in a northeast Asian population (Son et al., 2015) but exactly how UNC93A affects the pulmonary function is still unclear. They focused on conserved endosomal pathway proteins during infection of Aedes aegypti and found that viral RNA accumulates in UNC93A-silenced mosquitoes (Campbell et al., 2011). However, no further studies on mammals have found involvement of UNC93A in immunity.

Furthermore, our group presented microarray data on mouse hypothalamic N25/2 cell lines subjected to amino acid starvation. Alterations in gene expression of several putative SLCs, including Unc93a (Perland et al., 2017a), as well as known SLCs belonging to the SLC2, SLC6, SLC7, SLC16, SLC38, and SLC40 families (Hellsten et al., 2017b). Several of the SLCs belonging to the SLC2, SLC6, and SLC38 family are known to be regulated by nutrient availability (Wertheimer et al., 1991; Nagamatsu et al., 1994; Kilberg et al., 2005; Dregonova et al., 2013) and it is reported that Slc38a2 contain amino acid response elements (AAREs) and is activated by transcription factor 4 (ATF4), which contribute to the amino acid dependent regulation observed for this particular transporter (Pali et al., 2004, 2006; Tanaka et al., 2005).

We aimed to investigate the expression profile on both protein and mRNA level of UNC93A since it has not been clarified. In addition, we also wanted to extend the starvation data already available for Unc93a. We have characterized the putative SLC UNC93A in mice, with focus on protein localization in the central nervous system and mRNA expression changes during different nutrition conditions. Using immunohistochemistry (IHC), we found UNC93A staining in cortex, hippocampus, part of the hypothalamus, but only observed in the fluorescent IHC, and cerebellum. UNC93A was found to be expressed mostly in neurons with staining located in close proximity to the plasma membrane. We also investigated changes in expression levels of Unc93a in response to amino acid deprivation in mouse embryonic cortex cells as well as study this in a mouse model, where mice were subjected to 24 h of starvation. Unc93a gene expression was found in brain areas and peripheral tissues in low to moderate levels in the adult mice and Unc93a was also found to change expression levels due to both amino acid deprivation in embryonic cortex cultures and after starvation in in vivo samples from mice. In addition, we studied the presence of promoter motifs and sensing elements within Unc93a and 1000 bases upstream of the transcription start site (TSS), were we found one TATA box and two ATF4 motifs.

RESULTS
UNC93A Is Evolutionary Conserved in Animalia and Has 12 Potential Transmembrane Helices

A Hidden Markov model (HMM) for UNC93A was built and used in the search for related sequences to the human UNC93A. The search was carried out in nine different proteomes; Bos taurus (bs), C. elegans (ce), Danio rerio (dr), Gallus gallus (gg), Homo sapiens (hs), Mus musculus (mm), Nomascus leucogenys (nl), Pan troglodytes (pt), and Rattus norvegicus (rn). The model revealed that UNC93A was present in all these species representing several mammalian branches (Table 1) and its closest relatives were, MFSD11 and UNC93B1, illustrated by a phylogenetic tree (Figure 1). Most similar to hsUNC93A were the orthologs found in P. troglodytes, the common chimpanzee, and N. leucogenys, the northern white gibbon. In addition, orthologs were present in common laboratory animals such as chicken, mice, and rats, making it possible to study protein expression and function. In mice, there are two orthologs, mmUNC93A and mmGM9992. Using EMBoss Needle (Li et al., 2015) protein alignments were performed to study protein similarities. Both proteins are similar to each other (99.6%) and to hsUNC93A, 71.9 and 72.1%, respectively.

The secondary and tertiary structures of hsUNC93A were modeled using Phyre2 (Kelley et al., 2015). Homology modeling for UNC93A found a possible structure with twelve transmembrane helices, a common number of transmembrane helices in MFS proteins (Madej et al., 2013), and one alpha helix present in the loop between residue 158 and 200 (Figure 2A). Similar to other structure models for putative SLCs (Perland...
et al., 2017b), UNC93A has the MFS loop present between amino acid 218 and 249. The N-terminus and the C-terminus are shorter (11 and 28 amino acids, respectively) compared to its related protein, UNC93B1 (approximately 60 amino acids or longer) (Perland et al., 2017a), however, the N- and C-termini can vary between SLCs of MFS type (Yan, 2015; Perland et al., 2017a,b). 89% of the residues (407 of the total 457 residues) were modeled with 100% confidence, but only 11% of the amino acid matched the template sequence. However, the high confidence indicates that UNC93A probably adopts the overall fold illustrated, but surface loops can deviate (Kelley et al., 2015). Predictions of the tertiary structure reveal a globular protein (Figure 2B), with helices packed to form a pore (Figure 2C, top view), reminiscent of other putative SLCs (Perland et al., 2017a,b). The rainbow gradient of the secondary and tertiary structures shows helices from N-terminal (dark blue) to C-terminal (red).

Verification of Antibodies for Protein Localization Studies

Two commercially available antibodies for UNC93A were tested for use in protein localization and were verified using Western Blot on protein samples from HEK293 cells. UNC93A has three known isoforms in human and one known isoform in mice (Cunningham et al., 2015), both orthologs have one N-linked glycosylation site as found by analysis via NetOGlyc 4.0 (Steentoft et al., 2013). Following chemiluminescent detection, one band for the C-terminal anti-UNC93A (ab69443, Abcam) at 46.2 kDa (Figure 3A) was seen, with the expected size at 50.3 kDa (MGI:1933250) (Cunningham et al., 2015) (Ensemble release 90). In addition, western blot was performed using C-terminal anti-UNC93A blocked with a peptide corresponding to the epitope recognized by the antibody (Figure 3B). The peptide blocked the signal from the C-terminal antibody. Beta-actin served as a control for loading and primary antibody specificity.

TABLE 1 | Related proteins identified using a Hidden Markov model for mammalian UNC93A.

| Species          | Annotated name | UniProt ID | Annotated name | UniProt ID | Annotated name | UniProt ID |
|------------------|----------------|------------|----------------|------------|----------------|------------|
| B. taurus        | btUNC93A       | F1MB34     | btUNC93B1      | E1BBH4     | btMFSD11       | F1MGS4     |
| C. elegans       | ceUNC93        | Q93380     |                |            |                |            |
| D. rerio         | drUNC93A       | A0A0A0MPU6 | drUNC93B1      | F1QYA8     | drMFSD11       | A0A0R4IKA7 |
| G. gallus        | gglUNC93A      | F1NVF3     |                |            |                |            |
| H. sapiens       | hsUNC93A       | Q669B7     | hsUNC93B1      | Q9H1C4     | hsMFSD11       | Q669B51    |
| M. musculus      | mmUNC93A       | Q710D3     | mm UNCO93B1    | E9PYK0     | mmMFSD11       | Q710D8     |
| Mm. musculus     | mmUNC93A       | Q710D3     | mm UNCO93B1    | E9PYK0     | mmMFSD11       | Q710D8     |
| N. leucogenys    | nlUNC93A       | G1RL06     | nlUNC93B1      | G1R3Z9     | nlMFSD11       | G1R3Z9     |
| P. troglodytes   | ptUNC93A       | H2QU16     | ptUNC93B1      | H2Q497     | ptMFSD11       | H2Q497     |
| R. norvegicus    | rnUNC93A       | M0RA42     | rnUNC93B1      | D3ZDJ4     | rnMFSD11       | D3ZDJ4     |

Non-fluorescent free floating immunohistochemistry on 70 µm thick brain sections were performed for UNC93A to provide an overview of the protein staining pattern throughout the adult mouse brain. Staining was found in cell bodies and projections (Figure 4), with especially intense signal in cortex (Figure 4BI), hippocampus (Figure 4BII) and cerebellum (Figure 4CII). No staining was seen in the hypothalamic and preoptic areas surrounding the third ventricle (Figure 4AI) or striatum (Figure 4AII). In cortex, the staining was visual in
FIGURE 1 | Phylogenetic analysis of UNC93A. Related protein sequences to the human UNC93A were found using a Hidden Markov model (HMM). UNC93A was evolutionary conserved in Caenorhabditis elegans (ce), Danio rerio (dr), Galus gallus (gg), Homo sapiens (hs), Mus musculus (mm), Nomascus leucogenys (nl), Pan troglodytes (pt) and Rattus norvegius (rn), and it was closely related to MFSD11 and UNC93B1.

FIGURE 2 | The subcellular localization of UNC93A in primary cortex cells. Fluorescent immunocytochemistry were performed on primary cortex cells, prepared from embryos at days 14–16, to determine the subcellular localization of UNC93A. Earlier studies have performed transfection with a clone expressing UNC93A and then concluded that UNC93A is a plasma membrane protein, however, no localization study using antibodies for UNC93A and subcellular parts has been performed previously. Double staining with the Pan Neuronal marker, which stains axons, dendrites, nucleus and cell body of the neurons, confirmed localization of UNC93A to neurons (Figure 7A), no co-localization was seen with KDEL (Figure 7B), a marker targeting the signal peptide.
for retention and retrieval of protein to the ER (Raykhel et al., 2007), Syntaxin 6 (Figure 7C), targeting the trans-Golgi network (Bock et al., 1997), or Synaptotagmin (Figure 7D), marking synaptic vesicles (Pevsner et al., 1994). Partial co-localization was observed with SNAP25 (Figure 7E), a protein important for vesicle fusion at the plasma membrane (Pevsner et al., 1994). Since the staining of UNC93A was rather modest, a negative control was run to examine the unspecific binding of the secondary antibodies, and no staining was detected for neither Alexa A488 donkey anti-rabbit nor Alexa A594 goat anti-mouse in the absent of primary antibodies (Figure 7F). In addition, anti-UNC93A (ab173552, Abcam) was run on primary cortex cells as well to investigate if the second antibody targeting the N-terminal of UNC93A gave similar staining pattern as the C-terminal antibody used for the protein localization studies. The signal for the N-terminal UNC93A antibody correspond well to the C-terminal UNC93A antibody signal (Supplementary Figure S1B).

**Unc93a Expression Is Altered after Amino Acid Deprivation in Mouse Primary Cortex Cells**

Many SLCs, both putative and known, are involved in metabolism (Nagamatsu et al., 1994; Palii et al., 2004; Hellsten et al., 2017b; Lekholm et al., 2017; Perland et al., 2017b), and the recently amino acid starvation was found to alter Unc93a levels in N25/2 hypothalamic cells in mouse (Perland et al., 2017a), the effect of nutrient deprivation on Unc93a was studied. Amino acids deprivation induced transient alteration of Unc93a expression in primary cortex cells (Figure 8A). Gene expression of Unc93a was analyzed using qPCR and the regulation was studied in mature embryonic cortex cells cultured in complete or limited amino acid media for 3, 7, and 12 h. After 3 h of amino acid deprivation, Unc93a expression was greater than (p = 0.0031) the expression in the control samples. After 7 h the gene expression in deprived and control cells were equal, and
FIGURE 3 | Antibody verification. UNC93A antibody (ab69443, Abcam) used for histological methods was verified using Western blot with a protein samples from HEK293 cells. (A) Staining with UNC93A gave one band at 45.2 kDa (expected size at 50.3 kDa). For specificity evaluation an UNC93A peptide (SBP4551, Sigma–Aldrich) that corresponds to the epitope recognized by the antibody was used to block the signal. (B) The signal was neutralized in the blocking peptide lane, while a clear band was displayed in the control lane. Beta-actin was run to visualize the amount of loaded protein. Specificity was also tested by performing IHC with and without a blocking peptide on 7 µm brain sections from mouse. The fluorescent intensity was measure using Image J, Fiji edition, and the intensity was corrected to the number of cells in the picture. The graphs represent the average intensity per cell (±SD) and expression differences were calculated using t-test (*p < 0.05, **p < 0.01, ***p < 0.001). (C) The peptide reduced UNC93A staining in the brain. For example the peptide blocked fluorescent intensity in both (D) cortex and (E) cerebellum.

after 12 h the expression of Unc93a was downregulated compared with controls (p = 0.0027).

Gene Expression Changes after Starvation in Adult Mice

As a change in gene regulation was detected in the primary embryonic cortex cultures, the gene expression was also analyzed from brain regions (Figure 8B) and peripheral tissue samples from adult mice subjected to 24 h starvation. Gene expression alterations were measured using qPCR and expression was compared with controls. 24 h starvation upregulated the expression of Unc93a in cerebellum (p = 0.0023), olfactory bulb (p = 0.0299) and spinal cord (p = 0.0229), while downregulated it in hypothalamus (p = 0.0359) compared with controls (Figure 8C). In the periphery, the mRNA expression of Unc93a was upregulated in intestine (p = 0.0319), lung (p = 0.0114), kidney (p = 0.0049), thymus (p = 0.0434) and heart (p = 0.0173) and downregulated in fat (p = 0.0213) (Figure 8D). As the gene expression was normalized to genomic DNA for Unc93a for each run, the expression levels can be compared and a clear difference can be seen in the expression between the controls in different tissues.

Unc93a had a low mRNA expression profile in the central nervous system, with highest expression in cerebellum, hypothalamus, hippocampus, and spinal cord (Figure 8C). In comparison, Unc93a was expressed abundantly in peripheral
tissues, with the highest mRNA expression in intestine, liver, spleen, testis, and fat (Figure 8D).

All qPCR was run three times and the graphs (Figures 8C,D) illustrate the merged relative mRNA expression of Unc93a and the p-values for all three runs are summarized in Figures 8E,F. All three runs followed similar mRNA expression pattern regarding expression level and alteration after 24 h starvation compared with controls.

The response observed for Unc93a indicates the presence of transcription factor recognitions motifs important during nutrient deprivation and starvation in the promoter of Unc93a and within the gene itself. The whole gene sequence and 1000 bases upstream of the TSS from mouse was investigated, using MEME suite and Eukaryotic promoter database, for promoter motifs (CAAT box and TATA box), three different sensing elements: The nutrient-sensing response unit (NSRU), consisting of NRSE-1 and -2 (Zhong et al., 2003), AARE, e.g., ASNS and CHOP that are found in Slc38a2 (Palii et al., 2004, 2006) and the transcriptional factor ATF4 and CCAAT-enhancer binding protein α (C/EBPα) and β (C/EBPβ) (Averous et al., 2004; Kilberg et al., 2005; Dey et al., 2012). Within the 1000 bases of Unc93a was the TATA box, while the CAAT box was not found. Neither the sequence for the NRSU ASNS nor CHOP AARE was found with in the region of interest. However, two copies of ATF4 motifs, approximately 100 and 150 bases upstream of TSS, as well as two copies of C/EBPα motifs, between 740 and 760 bases upstream of TSS, and one C/EBPβ motif, at 740 bases upstream of TSS, were found.
FIGURE 5 | Fluorescent immunohistochemistry of UNC93A and NeuN in the mouse brain. 7 µm paraffin embedded brain sections from adult mouse were stain for UNC93A (green) and NeuN (red) to study the co-localization to neurons. Staining was found in most parts and layers of cortex, e.g., (A) motor cortex and (B) piriform cortex, as well as (C) CA3 in the hippocampus, (D) hypothalamus and (E) cerebellum. A majority of UNC93A positive cells were also stained with the neuronal marker NeuN, except the Purkinje cells, a GABAergic nerve cell, in cerebellum that does not stain with NeuN. The nuclear marker DAPI (blue) is seen in all merged pictures. Scale bars represent 20 µm.

MATERIALS AND METHODS
Identification of Proteins Related to UNC93A and Structural Modeling
A Hidden Markov Model (HMM) was built for mammalian UNC93A proteins using the HMMER package (Eddy, 2011), as described in (Perland et al., 2017b). The HMM was utilized in the search from orthologs to UNC93A by scanning data sets listed in Table 2. Proteomes were obtained from Ensembl (Cunningham et al., 2015). The results were manually curated, where splice variants and pseudo genes were removed. The identified entries were combined in a multiple PSI/TM toffee sequence alignment (Notredame et al., 2000), before establishing their phylogenetic relations according to the Bayesian approach (Huelsenbeck et al., 2001). For detailed description, see (Perland et al., 2017b). In
short, the analysis was run via the Beagle library (Ayres et al., 2012) on five heated and one cold chain. It was performed with two runs in parallel \( (n \text{ runs} = 2) \), for a maximum of 2,000,000 generations.

Models of the human UNC93A structure was predicted using Phyre2 (Kelley et al., 2015). The UNC93A structure was modeled against the template with highest confidence, sequence coverage and identity. The glucose transporter from the bacteria
Staphylococcus epidermidis (PDB id c4ldsB) (Iancu et al., 2013), with 100% confidence and 89% amino acids sequence coverage, were used. Phyre2 provided both the secondary and tertiary predictions. Homology models were modified in Jmol: an open-source Java viewer for chemical structures in three dimensions\(^1\) (Hanson et al., 2013).

**Animals**

The study was approved by the Animal Ethics Committee in Uppsala, Sweden (Permit Number C419/12, C39/16, and C67/13) and is in accordance with the guidelines of European Communities Council Directive (2010/63). Male C57Bl6/J mice (Taconic M&B, Denmark) were used and considered as wildtype. The mice were housed in an ambient room temperature of 22°C with a 12 h light:dark cycle. All animals were provided food (standard chow R3, Lantmännen, Sweden) and tap water ad libitum, unless otherwise stated. All procedures were planned and executed to minimize the suffering and euthanasia was performed during the light period by either transcardiac perfusion or cervical dislocation.

**Western Blot**

Total protein concentration on protein samples were analyzed using a Coomassie Protein Quantification Kit (Sigma–Aldrich) and compared to a standard curve of Bovine Serum Albumine (BSA) included in the kit. Western blot was performed as described in (Perland et al., 2016). In short, protein samples, extracted using RIPA buffer (Sigma–Aldrich) supplemented with complete protease inhibitor (Roche) and phosphatase inhibitors (Merck Millipore), from HEK293 cell cultures (1150 µg/ml) or mouse brain tissue (6000 µg/ml) extracted according to (Hellsten et al., 2017a) were diluted 4:1 in Laemmli Sample Buffer (Bio-Rad) with 2-mercaptoethanol (Fluka), and loaded onto a 10% TGX Mini-protean gel (Bio-Rad). Gel separation at 200V for 30 min was performed before blotted onto PVDF membrane using the TurboBlot system (Bio-Rad). As reference a molecular weight marker was used (Prestained dual color, Bio-Rad). The membrane was blocked in 5% milk (Bio-Rad) diluted in TTBS for 1 h before two antibodies for UNC93A were used: Anti-UNC93A (1:100, rabbit, ab69443, Abcam) or Anti-UNC93A (1:80, 1:100, rabbit, ab173552, Abcam) incubation overnight at 4°C. For antibody specificity evaluation an UNC93A peptide that corresponds to the epitope recognized by the antibody was used to block the signal. Here, anti-UNC93A (ab69443, Abcam) was pre-incubated with UNC93A peptide (SBP4551, Sigma–Aldrich) in excess (5:1 to antibody concentration) in room temperature for 1 h prior the incubation. After 3 min × 10 min wash with TTBS, the membrane was incubated at room temperature for 60 min with HRP coupled secondary antibodies, HRP goat α-rabbit, diluted 1:10000 in milk blocking solution (Invitrogen). The membrane was developed using Clarity Western ECL Substrate (Bio-Rad) and the staining was visualized using a CCD camera (Bio-Rad). The staining was compared with the molecular weight marker using Image Lab software v5.2.1 build 11 (Bio-Rad). After detection for the peptide blocked and control membranes, the amount of protein loaded was quantified using beta-actin. Membranes incubated in Beta-actin (Sigma–Aldrich), diluted 1:10000 in 5% milk block, before 3 min × 10 min wash in TTBS and incubation with secondary antibody, HRP chicken α-mouse, diluted at 1:10000 in milk blocking solution (Invitrogen). The membranes were then again developed.

**Collection and Sectioning of Tissue for Non-fluorescent Immunohistochemistry**

Male C57Bl6/J mice (Taconic M&B, Denmark) were anesthetized with an intraperitoneal injection of 0.25 ml pentobarbital
mRNA expression changes of Unc93a in cell cultures and tissue samples from mice. The mRNA level of Unc93a in mouse embryonic cortex cultures subjected to amino acid deprivation was monitored after 3, 7, and 12 h. Normalization was performed against three housekeeping genes (Gapdh, H3a, and Actb) and the relative mRNA expression was compared with the controls, set to 100%, for each time point. Expression differences were calculated using t-test (*p < 0.05, **p < 0.01, ***p < 0.001). (A) Unc93a expression in amino acid deprived cells (AA–, orange) as compared with control cells (AA+, white with orange boarder). Alterations can be seen after 3 and 12 h of amino acid deprivation. (B) Picture of mouse brain illustrating the dissected areas used for gene measurement in the adult mice. (C) Brain tissue from brainstem, cerebellum, cortex, hypothalamus, hippocampus, olfactory bulb, pituitary gland, striatum, thalamus and eye as well as the spinal cord was taken from mice placed on 24 h starvation (n = 5, pooled sample). Normalization was performed to stable housekeeping genes and the qPCR was repeated three times. The merged relative mRNA expression (±SEM) was plotted and the expression level monitored in genomic DNA was set to 100% and the normalized quantities were compared to the genomic DNA. Changes were seen in cerebellum, olfactory bulb, spinal cord and hypothalamus. (D) Relative mRNA expression of Unc93a in peripheral organs. Alterations were observed in intestine, lung, kidney, thymus, heart and fat tissue. Discrepancies were noticed between the three runs, but all runs followed similar mRNA expression pattern regarding expression level and alteration after 24 h hours compared with controls. The p-values for all three runs are summarized for (E) brain regions and spinal cord as well as (F) peripheral organs.

sodium (Apoteksbolaget, Sweden). Transcardiac perfusion was performed using phosphate-buffered saline (PBS) (137.0 mM NaCl, 2.70 mM KCl, 8.10 mM Na2HPO4) followed by 4% formaldehyde (HistoLab, Sweden) fixation. The brains were dissected and stored in 4% formaldehyde at 4°C overnight. The next day, the brains were washed twice in Tris-buffered saline
(TBS) (0.04 M Trizma HCl, 0.01 M Trizma base, 0.15 M NaCl, pH 7.4) followed by 4% agarose gel (VWR, Germany) embedding. 70 µm coronal brain sections were cut with a vibratome Leica VT 1200 S (Leica Microsystems, Germany).

### Non-fluorescent Immunohistochemistry on Free-Floating Mouse Brain Sections

All chemicals were purchased from Sigma–Aldrich, unless otherwise stated. 70 µm brain sections were washed in TBS 4 min × 8 min before and after 10 min incubation in 10% MeOH, 3% H₂O₂ (Merck) in TBS. Sections were incubated in 1% blocking reagent (Roche Diagnostics) for 1 h followed by incubation in anti-UNC93A (ab69443, Abcam) diluted 1:200 in supermix (0.25% gelatin, 0.5% Triton X-100 in TBS) at 4°C overnight. Sections were rinsed in TBS 2 × 1+4 min × 8 min followed by incubation in secondary antibody [biotinylated goat-anti-rabbit IgG (H+L), Vector laboratories] diluted 1:400 in supermix for 1 h at room temperature. Sections were rinsed in TBS 5 min × 8 min before and after incubation in avidin Biotin complex kit [Reagent A, Reagent B (Vecstain, Vector Laboratories)] diluted 1:800 in supermix for 1 h. Immunostaining was visualized by incubating sections in 0.08% 3.3 Diaminobenzidine tetrahydrochloride (DAB), 0.20% NiCl₂ and 0.035% H₂O₂ in TBS followed by 4 min × 5 min TBS washes. Sections were mounted on gelatinized microscope Superfrost Plus slides (VWR) and dehydrated, 5 min in 70% and 95% EtOH, 10 min in 100% EtOH (Solveco) and 20 min in Xylene. Slides were rinsed in TBS 5 min before and after 10 min incubation in 10% MeOH, 3% H₂O₂ (Merck) in TBS. Sections were incubated in DPX Mountant for histology (Sigma–Aldrich) with micro cover slides (Menzel Gläser). Sections were analyzed using a Mirax Panoramic midi scanner with the Panoramic software version 1.15.4 RTM (3DHistech). The brightness of the images was adjusted to 75% before compiling the figure.

### Fluorescent Immunohistochemistry of Paraffin Embedded Mice Brain Sections

Brain tissue for immunohistochemistry was collected as described in Roshanbin et al. (2014). Fluorescent immunohistochemistry on paraffin embedded mouse brain sections was performed as described in (Perland et al., 2016) with antibodies all diluted in supermix for anti-UNC93A (ab69443, Abcam, 1:100), NeuN (Merck Millipore, 1:200) GFAP (Merck Millipore, 1:800) and nucleus marker DAPI (Sigma–Aldrich) diluted 1:15000 in PBS. For peptide blocked control section, UNC93A antibody (1:100) was pre-incubated with the corresponding peptide (SBP4551, Sigma–Aldrich) according to procedure described for western blot. Images were acquired using Olympus microscope BX55 with an Olympus DP73 camera and the cellSens Dimension v1.14 (Olympus) and images were then handled using ImageJ, Fiji edition (Schindelin et al., 2012). The differences in fluorescent intensity between the control sections and peptide blocked sections were quantified using ImageJ, Fiji edition. In short, pictures were taken with the same exposure time and the fluorescent intensity for the image was measured and then corrected against amount of cells. The general UNC93A staining in two sections was measured and the staining in cortex and cerebellum was measured in several pictures covering the whole structure. In the graphs, the fluorescent signal is presented as mean ± SD. For statistical analyses, GraphPad Prism software v 5.02: T-tests were performed for gene expression alteration where *p < 0.05, **p < 0.01, ***p < 0.001.

### Fluorescent Immunocytochemistry on Mouse Primary Cortex Cultures

Wildtype male and female mice were mated and at e14-15 the females were euthanized with cervical dislocation, the embryos removed and cortex dissected out and primary cultures were set up as previously described in Perland et al. (2016). Immunocytochemistry was performed as described in Perland et al. (2016) with anti-UNC93A diluted 1:100 in supermix blocking solution. Co-staining with neuronal marker Pan diluted 1:200 (MAB2300, Millipore), KDEL markers (ab12223, Abcam) diluted 1:200, Syntaxin 6 (Ab12370, Abcam) diluted 1:100, Synaptotagmin (ab13259, Abcam) diluted 1:200 and SNAP25 (ab25737, Abcam) diluted 1:100 in supermix blocking solution. Images were acquired at the SciLifeLab BioVis Facility (Uppsala University) using confocal LSM710 SIM (Zeiss) and the Zen black software (Zeiss) or Olympus microscope BX55 with an Olympus DP73 camera and the cellSens Dimension v1.14 (Olympus). Images were then handled using ImageJ, Fiji edition (Schindelin et al., 2012).

In addition, immunocytochemistry for the N-terminal anti-UNC93A antibody (ab173552, Abcam), diluted 1:80 in 5% milk block (Bio-Rad), was performed as described above.

### Amino Acid Deprivation in Primary Cortex Cells and Starvation Experiment in Adult Mice

To evaluate if response to amino acid availability alter gene expression of Unc93a, primary embryonic cortex cultures were partial deprived of common amino acids as described in Lekholm et al. (2017). In short, EBSS base media (Gibco) containing 1.0 mM Sodium-Pyruvate, 1% Penicillin-Streptomycin, 2% B27, 4X MEM Vitamin solution (Gibco), 10.9 mM HEPES buffer solution was prepared and amino acids L-arginine, L-cysteine,
L-lysine, L-methionine, L-phenylalanine, L-proline, L-threonine, L-tryptophan, and L-tyrosine (Sigma–Aldrich) were added. The control media was made as above but also contained additional 2.0 mM GlutaMax (Gibco) and glycine, L-alanine, L-asparagine, L-histidine, L-isoleucine, L-leucine, L-serine, and L-valine (Sigma–Aldrich). At culture day 10, the normal primary culture media, Neurobasal A was aspirated and the amino acid deprived media and control media was added instead. Hence, the cells were subjected to deprivation of glycine, L-alanine, L-asparagine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-serine, and L-valine (Sigma–Aldrich). The primary embryonic cortex cells were cultures in control media or deprivation media for 3, 7, and 12 h before total RNA extraction, cDNA synthesis and qRT-PCR were performed as detailed below.

Diet experiments were performed as described in Perlard et al. (2016) and material was used here to analyze the gene expression pattern of Unc93a. In short, adult male mice were starved for 24 h, while having access to water, before euthanasia and dissection, while control mice were kept on standard chow (5% fat, 21% protein, 51.5% carbohydrates). Central and peripheral organs from four mice were dissected and RNA extraction, cDNA synthesis and qRT-PCR were implemented as detailed below.

**Tissue Sampling, RNA Extraction and cDNA Synthesis**

Tissues from wildtype and starved mice were prepared and set up as described in Perlard et al. (2016). All tissues were stored in RNA later (Qiagen, Sweden) for 2 h at room temperature before placed at -80°C until total RNA was extracted.

Total RNA was extracted using Absolutely RNA Miniprep kit (Agilent Technologies) for mouse tissue samples or RNAasy Midi kit (Qiagen) for cells samples, according to the manufacturer's instruction and concentrations were measured using ND-1000 spectrophotometer (NanoDrop Technologies). The cDNA synthesis was performed using the Applied Biosystems High Capacity RNA-to-cDNA kit (Invitrogen) following manufacturer’s recommendations. 2 µg RNA template was used for the reaction and the cDNA was pooled and diluted to 5 ng/µl as described in (REF - MFSD5 and MFSD11 manuscript).

**Primer Design and Quantitative Real-Time PCR (qRT-PCR)**

All primers were designed using Beacon Design 8 (Premier Biosoft). Unc93a (accession no: NM_199252.2) forward 5′-tc cactggtgctcatcct-3′ and reverse 5′-aggagaagcacaaga-3′. Reference housekeeping genes: beta tubulin 4B (βTub) forward 5′-agttgtctccctcaacag-3′, reverse 5′-tacctggctgaatgc-3′, ribosomal protein L19 (Rpl19) forward 5′-aatgcgaatccacac tc-3′, reverse 5′-gaagtggacatcagg-3′, histone cluster 1 (H3a) forward 5′-ctctgttgttgtgtttg-3′, reverse 5′-cattgga tgtcctgag-3′, Peptidylprolyl isomeras A (Cyclo) forward 5′-tt tggagggaaagaaag-3′, reverse 5′-acagaagaaatgttgtg-3′ and actin-related protein 1B (Actb) forward 5′-cccttggtgtg atggatcctgctg-3′, reverse 5′-cagactgtgttggctatagg-3′.

Unc93a gene expression and expression alteration were determined using qRT-PCR. Final volume for each reaction was 20 µl containing 3.6 µl 10X DreamTaq buffer (Thermo Fisher Scientific), 0.2 µl of 25 mM DNTP mix (Invitrogen), 1 µl DMSO, 0.5 µ SYBR Green (1:10000, Invitrogen) in 1XTE buffer (pH 7.8), 0.08 µl DreamTaq polymerase (5 U/µl, Thermo Fisher Scientific), 0.05 µl of forward and reverse primer (100 pmol/µl) and 5 µl cDNA (10 ng/µl) for brain and peripheral tissues or 2 µl cDNA (30 ng/µl) for primary embryonic cortex cells. The volume was adjusted with sterile water. An iCycler real-time detection instrument (Bio-Rad) was used with following settings: initial denaturation for 30 s at 95°C, 55 cycles of 10 s at 95°C, 30 s at 55°C for housekeeping genes and 59°C for Unc93a amplification and 30 s 72°C. A melting curve was initiated for 81 cycles with 10 s interval, starting at 55°C and the temperature increased 0.5°C per cycle. All qRT-PCR was run three times with samples in triplicates, negative control and genomic DNA (10 ng/ul) were included on each plate.

**Analysis of qRT-PCR and Statistics**

All data was collected using MyIQ software (Bio-Rad Laboratories). Primer efficiency was calculated using LinRegPCR software v7.5, followed by Grubbs Outlier test (GraphPad software) to remove outliers before calculations based on primer efficiency. The GeNorm protocol (Vandesompele et al., 2002) was used to determine stable reference genes before calculating the geometric mean of the reference genes, which was then used to normalize the gene expression of Unc93a. For primary embryonic cortex cultures the gene expression was compared to the controls, set to 100%, for each time point. For brain regions and peripheral tissues samples the genomic DNA was set to 100% and the normalized quantities were thereafter compared to the genomic DNA. Graphs represent merged values for three runs and are presented as means ± SEM. For statistical analyses, GraphPad Prism software v 5.02: T-tests were performed for gene expression alteration where *p < 0.05, **p < 0.01, ***p < 0.001.

**Analysis of 1000 Bases Upstream of Transcription Start Site and Unc93a**

The presence of promoter, transcription factor and sensing elements motifs for mouse Unc93a (Accession number: MGI:1933250) and 1000 bases upstream of the TSS were predicted using the MEME suite (Bailey et al., 2009) and Eukaryotic Promoter Database (Cavin Perier et al., 1998). Two promoter motifs, the TATA box and CAAT box, as well as three sensing elements, the Nutrient-Sensing Response Unit (NSRU), which contain the Nutrient-Sensing Response Elements 1 and 2 (NSRE1 and 2) (Zhong et al., 2003), AARE like ASNS and CHOP AAREs that are found in SLC38A2 (Palli et al., 2006), and the transcription factor Activating Transcription Factor 4 (ATF4) and CCAAT-enhancer binding protein α (C/EBPα) and β (C/EBPβ), were analyzed.

**DISCUSSION**

As mentioned earlier transporter proteins such as solute carriers are underrepresented in research (Cesar-Razquin et al., 2015), however, lately there have been an increase in research regarding
novel SLCs (Perland et al., 2016, 2017b; Hellsten et al., 2017a; Lekholm et al., 2017). Proteins containing major facilitator superfamily motifs (e.g., MFSD1-14B, UNC93A, and B1, SV2s), which are phylogenetically related to SLCs of MFS type, are, today, suggested to be putative SLCs (Perland and Fredriksson, 2017; Perland et al., 2017a). Here, we have begun to characterize UNC93A, which is one of the proteins that phylogenetically cluster with SLCs of MFS type (Perland and Fredriksson, 2017), but is not similar enough to any known SLC to be grouped into any of the 52 existing SLC families. In humans, UNC93A was found to be most closely related to UNC93B1, a transporter like protein important for regulation and trafficking of toll-like receptors (Lee et al., 2013; Huh et al., 2014), and to the putative SLC MFSD11 (Perland et al., 2016). A Hidden Markov model (HMM) for UNC93A was built and we search for orthologs present in C. elegans, in which the Unc-93 protein was first discovered, and mammalian genomes. We found that UNC93A is evolutionary conserved in mammals and its most closely related proteins were UNC93B1 and MFSD11. In addition, the secondary and tertiary structures were predicted for the human protein. UNC93A was modeled based on a glucose transporter in S. epidermis, and found to have 12TMS, similar to other MFS proteins sharing the same evolutionary origin (Madej et al., 2013; Yan, 2015).

Dahlin et al. (2009) studied the expression of 307 Slc genes in the Allen Brain Atlas (Dahlin et al., 2009). UNC93A was not one of the 307 Slc genes included in the screen, and our panel adds information to the expression pattern of transporters in the mouse brain. Co-localization to neurons was verified as UNC93A localized to many cells stained with a the marker NeuN (Mullen et al., 1992), but not with cells positive for the astrocytic marker GFAP. The expression is localized to the cell body of neurons and the projections in cortex, hippocampus and the Purkinje cells. The strong signal present in Purkinje cells and parts of the hippocampus, which is rich in inhibitory neurons (Wheeler et al., 2015; Hamilton et al., 2017), point to that some UNC93A is located to inhibitory neurons. However, additional verification by co-localization studies with neuronal subtype markers is needed to verify the neuronal type UNC93A is expressed in. Earlier work performed by Liu et al. (2002) reported UNC93A to be a plasma membrane protein. Here the intracellular localization was studied with markers for ER, Golgi, Synaptic vesicles and plasma membrane in embryonic cortex cells. Similar to the staining observed in the adult mouse sections, UNC93A staining was seen in cell bodies and the staining was evenly distributed over the cell (Figure 7). Once again the co-localization with the Pan neuronal marker indicated a neuronal localization for UNC93A, but pin-pointing the subcellular localization of UNC93A was challenging. UNC93A did not co-localize to intracellular markers for ER, Golgi or synaptic vesicles. Furthermore, UNC93A only partially co-localized with SNAP25, here used as a membrane marker. Taken together, UNC93A was localized close to the plasma membrane, as reported earlier, however, it was not located to the plasma membrane exclusively and the possibility that UNC93A can be expressed in other types of vesicles or organelles that have not been stained for in this study cannot be ruled out. The partial overlap observed between UNC93A and Pan neuronal marker and SNAP25 antibody, as well as the granular staining of UNC93A, highlights the possibility that UNC93A, similar to UNC93B1 is involved in trafficking of proteins. The ortholog to UNC93A in C. elegans is believed to be one out of five genes identified in the screen which regulates muscle contraction (Levin and Horvitz, 1992; de la Cruz et al., 2003), and it is suggested to function as a subunit of a potassium channel, SUP-9 which is similar to the human TWIK related acid-sensitive K+ (TASK) channels. These channels are important for resting membrane potential, action potential duration and modulating the responsiveness to synaptic input (Duprat et al., 2007). de la Cruz et al. (2003) reported that SUP-9 was similar to the human TASK-1 and TASK-3 (de la Cruz et al., 2003), but there is also a Tandem Pore domain K+ Channel subunit (Karschin et al., 2001), TASK-5, that is related to SUP-9 found when we searched for homologs using NCBI homologene\(^2\) or phylomeDB\(^3\). It is therefore possible that UNC93A together with TASK channels are needed for controlling the potassium flow in neurons. TASK-1 and TASK-3 are widespread in the rat brain, but no expression was detected in, e.g., the Purkinje cells (Karschin et al., 2001; Medhurst et al., 2001; Marinc et al., 2014), where we observe extensive UNC93A staining. The expression pattern of TASK-5 in the mouse brain is low with expression located mostly to the auditory system, olfactory bulb mitral cells and the Purkinje cells (Karschin et al., 2001). The differences in protein expression patterns for UNC93A and the TASK channels point to the possibility that UNC93A is not linked to the TASK channels.

Nutrients (glucose, amino acids, and lipids) are simple compounds needed to produce energy and cellular biomass and, due to their importance there are distinct mechanisms to sense intracellular and environmental levels (Efeyan et al., 2015). During starvation both levels of nutrients and ion transport are reduced (Hamwi et al., 1967; Zhao and Willis, 1988). This initiate integrated biochemical and physiological changes that affects not only organs involved in absorption, distribution, metabolism and excretion (ADME) but the whole body energy requirement is usually reduced, which results in lower O\(_2\) consumption and CO\(_2\) production (Ehrlich et al., 2016). In addition, processes to acquire nutrients are initiated that requires signaling cascades and membrane-bound proteins and enzymes (Campbell, 2007; He et al., 2009; McCue, 2010). Alterations in transcription due to nutritional changes is of interest since patient suffering from various diseases also have effects on metabolism, e.g., patients with anorexia (Heilbronn et al., 2007) and obesity (Barnes et al., 2011) as well as neurodegenerative diseases such as Alzheimer’s, Parkinson’s, and Huntington’s disease (Cai et al., 2012). The gene expression of Unc93a in mice has not, to our knowledge, been studied previously and mRNA was found in the central nervous system and peripheral tissues. The protein localization and the mRNA expression corresponded in the brain. Earlier, our group reported expression changes in Unc93a gene expression in N252 hypothalamic mouse cells subjected to amino acid starvation, where the mRNA was induced after the first 5 h and downregulated at 16 h (Hellsten et al., 2017b; Perland

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\(^2\)https://www.ncbi.nlm.nih.gov/homologene

\(^3\)http://www.phylomedb.org/
et al., 2017a). Building on this, we studied the gene expression alterations in vitro and in vivo. In mouse embryonic cortex cells subjected to amino acid deprivation, the same transient alteration was observed as in the N25/2 hypothalamic mouse cell line, where at 3 h Unc93a gene expression was upregulated, followed by a reduction in expression at 12 h. Furthermore, we studied Unc93a expression in mice subjected to complete starvation for 24 h, where we observed increased mRNA in peripheral tissues such as intestine and kidneys, both important for ADME of substrates, as well as lungs, thymus and heart. The effect in the central nervous system was limited to cerebellum, hypothalamus, olfactory bulb and spinal cord.

The main source of energy for nerve cells and the body as a whole is glucose, although, amino acids, especially essential amino acids that cannot be produced by the cell itself, are needed for metabolic processes and for cell-to-cell signaling (Mergenthaler et al., 2013; Hellsten et al., 2017b). Furthermore, a steady state of the ion balance is important to maintain to ensure proper cell signaling cascades, membrane potentials, enzyme activation and co-transport of different nutrients (Halperin and Kamel, 1998; Duprat et al., 2007; Framod et al., 2013; Hellsten et al., 2017a). The mRNA expression changes observed from Unc93a does not follow other studied putative transporters, e.g., Mfsd1, Mfsd3, Mfsd14a, and Mfsd14b (Lekholm et al., 2017; Perland et al., 2017b). None of the transporters were upregulated by amino acid starvation in the hypothalamic cell cultures (Perland et al., 2017a), however, Mfsd1, Mfsd14a, and Mfsd14b was upregulated during amino acid deprivation in the primary cortex cells after 3 h, but they stayed upregulated (Mfsd1) or were not affected at all after 12 h (Mfsd14a and Mfsd14b). In mice subjected to starvation, similar transcript changes were observed between the studied putative transporters and Unc93a in some of the tissues such as upregulation in cerebellum and downregulation in hypothalamus (Lekholm et al., 2017; Perland et al., 2017b), although the peripheral organs has not been studied for the other putative transporters.

Amino acid starvation in the N25/2 mouse hypothalamic cell culture increased the expression of several genes important for different pathways in the cell. An early induction in genes involved in amino acid transport and transcription regulation are seen probably in an attempt to increase the uptake of the missing biomass (Efeyan et al., 2015; Hellsten et al., 2017b). The early induction on mRNA expression in the two in vitro experiments points to the possibility that Unc93a has an early role in the response to energy availability in these two types of cell cultures and maybe Unc93a has a role in sensing the nutrient limitation, energy reduction or amino acid uptake similar to the sodium dependent amino acid transporter Slc38a2. An early induction on transcript level during amino acid starvation is typically seen for Slc38a2 (Pali et al., 2004; Tanaka et al., 2005; Hellsten et al., 2017b), which is known to be regulated by a set of transcription factors, such as ATF4 and C/EBP, that binds to the AAREs present in the first intron of the gene (Pali et al., 2004). ATF4 is a master regulator that is crucial for adaptation to stresses by regulating genes, involved in metabolism, nutrient uptake, antioxidative stress and apoptosis, by binding to the AARE (Kilberg et al., 2005; Dey et al., 2012). Similar C/EBP is a transcription regulator important for genes involved in metabolism, where C/EBPα has a more general role and C/EBPβ is more involved in fine-tuning responses (Ramji and Foka, 2002). When analyzing the promoter region for mouse Unc93a, both motifs for ATF4 and C/EBP were found. The Unc93a transcription level induction observed in vitro and in vivo are probably caused by the presence of ATF4 and C/EBP motifs in the promoter. However, the NSRU and the AAREs ASNS and CHOP could not be found in the promoter or Unc93a sequence obtained from the mouse genome. Furthermore, the mRNA induction for many amino acid transporters, for example Slc38a2, remains upregulated after a longer period of amino acid starvation and deprivation (Hellsten et al., 2017b), which is not seen for Unc93a, indicating that it is involved in alternative steps within the same pathway or another pathway. Other in vivo and in vitro studies have reported that Slc2a1 (GLUT1) and Slc2a3 (GLUT3) expression are induced after 72 and 48 h, respectively, of starvation in mammals (Wertheimer et al., 1991; Nagamatsu et al., 1994). The induction observed in the mouse brain for the Slc2a3 was limited to cortex and hippocampus; two structures in which the transcript level of Unc93a were not affected after starvation. In addition, only two members of the SLC2 family were regulated by amino acid starvation in the hypothalamic cell culture, Slc2a1 (downregulated) and Slc2a12 (upregulated) during all time-points (Hellsten et al., 2017b), indicating that Unc93a is probably not involved in similar pathways as the SLC2 family.

ATF4 is not only a master regulator of genes involved in metabolic processes it is also essential for stress-induced autophagy, a process where macromolecules and organelles that are not needed are degraded to provide nutrients (Huett and Xavier, 2010; Benjamin et al., 2013; Tan et al., 2017). The increase in transcript levels of Unc93a observed in vitro and in vivo might indicate that it is involved in autophagy to provide the body with needed macronutrients during starvation, and it is possible that Unc93a is involved in the initiation of autophagy. Furthermore, the alterations observed for Unc93a expression in hypothalamus and olfactory bulb, two organs important for feeding (van den Top et al., 2004; Caba et al., 2014; Betley et al., 2015) further highlight its role in metabolism.

UNC93A has been reported to be expressed on the plasma membrane (Liu et al., 2002) and therefore one can assume that UNC93A can be important for sensing or transportation of nutrients and/or ions or, similar to UNC93B1, be involved in regulating and trafficking of membrane-bound proteins to, e.g., the plasma membrane to increase the membrane permeability for nutrients and/or ions. The same transient regulatory pattern seen for Unc93a in the hypothalamic cell culture from mice is also observed for other ion transporters (Montalbetti et al., 2013; Hellsten et al., 2017b), especially the iron transporter Slc40a1. The fact that Unc93ais regulated in a similar fashion as Slc40a1 (Hellsten et al., 2017b) and is needed together with the TASK channels in C. elegans to regulate ion flow in the muscles (de la Cruz et al., 2003), make it possible that UNC93A is important to keep the ion levels steady in the cell during normal conditions and challenges, e.g., it might regulate ion inflow important for cellular...
pH, antioxidant stress responses and osmolality either directly or indirectly by regulating other transporters.

A multicellular model system does not react to nutrient scarcity as a unicellular system since homeostatic responses aimed to maintain the circulating nutrient levels are available as well as storage capacity (Efeyan et al., 2015; Ehrlich et al., 2016). The transient alteration, early upregulation and later downregulation, observed in cell cultures and the late upregulation observed in adult mice can be due to the differences in the setup of starvation on amino acids vs. complete food starvation. It could also possibly be due to the fact that an animal have other processes, storage and organs contributing to energy homeostasis during starvation, which the hypothalamic cell line and primary cortex cultures lack. However, all of the results point to Unc93a having a vital and general function in cellular metabolism, either by regulating the nutrient, end-product or stress level. However, it is difficult to pin-point the exact role of UNC93A in these processes that occurs during different nutrient availabilities. In adult mice the intentional experiment was to monitor changes due to nutrient availability, but as other parameters such as stress and immune responses were not measured, the gene regulation seen could be due to other confounding factors and not just energy metabolism. It is therefore possible that the induction of Unc93a is caused by stress and immune responses, which can be further strengthened since UNC93A has ATF4 motifs present in its promoter, a transcription factor reacting to integrated stress responses, UNC93A has been reported to be important for viral assembly in mosquitos (Campbell et al., 2011) and its closest related protein, UNC93B1, is highly involved in the innate immunity (Lee et al., 2013; Huh et al., 2014).

CONCLUSION

(I) UNC93A is a protein with 12 transmembrane helices, that fold similar to other MFS and (II) UNC93A is expressed in brain and peripheral tissues. (III) Unc93a has ATF4 and C/EBP motifs present in the promoter region which probably regulate its transcription during nutritional challenges. (IV) The response to amino acid availability in vitro indicates a role in sensing nutrient limitation, changes in energy, autophagy or transportation of nutrients. (V) The similar transcript change observed between Unc93a and Slc40a1 also point to the possibility that Unc93a is important for ion regulation during cellular challenges such as starvation and/or stress. (VI) Taken together Unc93a seem to have a vital and general role in energy homeostasis and cellular metabolism. Even though the function and the substrate profile of UNC93A are still unclear, the specific histological expression of the transporter both in central nervous system and peripheral tissues makes this transporter interesting to study further. The gene regulation results observed for Unc93a both in vitro and in vivo, due to nutrient availability, is a reason to perform more research as it is clearly involved in regulation and/or sensing of important cellular pathways and mechanisms, providing a clear example why transporters are important to study further.

AUTHOR CONTRIBUTIONS

MC: Designed experiments, prepared material for qRT-PCR and performed immunohistochemistry, immunocytochemistry, qRT-PCR, and analysis, set up primary cultures, imaging, compiled figures and tables and drafted the manuscript. EL: Set up starvation experiments and primary cultures, performed WB on HEK293 samples, aided in analysis of results, imaging of fluorescent immunohistochemistry on sections and primary cultures, and wrote parts of the manuscript. SH: planned and performed deprivation experiments in primary cell cultures, prepared tissue, performed non-fluorescent DAB IHC, imaging and compiled figure for DAB, wrote parts of the material and methods, assisted in analysis of qRT-PCR and assisted in WB. EP: Set up starvation experiments, performed proteomic analysis, compiled tables and wrote parts of the section “Material and Methods.” RF: Designed the project, performed proteomic analysis and aided in data analysis and drafted the manuscript. All authors have read and approved of the manuscript and helped with interpretation of results.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnmol.2017.00351/full#supplementary-material

FIGURE S1 | Verification and protein localization of a N-terminus anti-UNC93A antibody. There are other antibodies on the market for UNC93A and the N-terminal antibody (ab173552, Abcam) was also verified by Western blot on a protein homogenate from mouse brain. Two concentration were tested and staining with N-terminal UNC93A gave two bands at approximately 52 and 35 kDa in (A) the supernatant (S2) but not the pellet (P1). (B) The N-terminal UNC93A (lower raw) was also compared to the UNC93A (ab69443) used in this paper (upper raw). The two staining's corresponded, but the N-terminal UNC93A was more nuclear. The N-terminal antibody was not further used in the protein localization study. UNC93A is in green and the nuclear marker DAPI is blue. Scale bars represent 20 μm.
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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