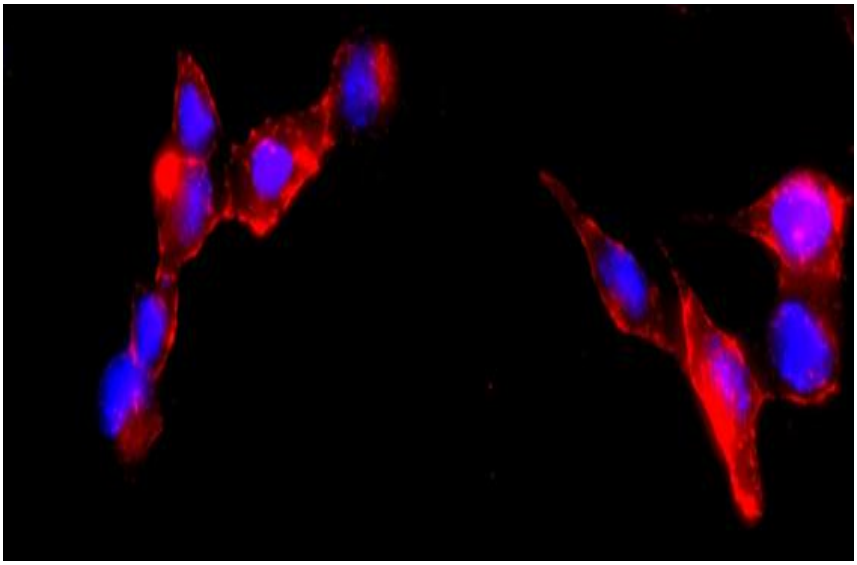
UPPSALA
UNIVERSITET

Characterization of solute carrier SLC38A6



Somar Al-walai

Molecular biotechnology/Bioinformatics engineering programme
Research training 15 hp, 2012
Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden
Supervisor: Sonchita Bagchi

Characterization of solute carrier SLC38A6

Somar Al-walai

Department of Neuroscience, Functional Pharmacology, Uppsala University, Uppsala, Sweden

Abstract
Transport across the membrane of a cell is of crucial importance for cellular functions. The solute carrier family, SLC38 is a family of membrane proteins that transports various substances through the membrane and thus performs many physiologically important functions, for example, transport of glutamine from astrocyte to neurons in the central nervous system. In this paper, we demonstrate that one of the transporters in this family named SLC38A6 forms several protein complexes with a variety of proteins in the membrane and in synaptic vesicles, suggesting that SLC38A6 is involved in the synaptic release of neurotransmitters in synapses. We performed sensitive protein interaction analysis between the protein of interest and a variety of proteins expressed at different sites in the neuronal cell. We showed that SLC38A6 interacts with proteins in the cell membrane as well as in the membrane of synaptic vesicles. The current theory is that SLC38A6 interact with these proteins when the synaptic vesicles are in close proximity with the cell membrane during the release of the neurotransmitters.

Introduction
The boundaries of cells as well as cell organelles are defined by their cell membrane. Approximately one third of the proteins from any organism are embedded in the cell membranes. These membrane proteins perform different cellular functions such as transport, enzymatic activity, signal transduction, cell to cell recognition, intercellular attachment and anchoring to the cytoskeleton. 30% of the proteins in the human genome are membrane proteins (Wu et al. 2003). It is of importance that the amino acid transport across the cell membrane as well as the mitochondrial and vesicular membranes is well-regulated (Hagglund et al. 2011). The solute Carriers (SLC:s) are the second biggest family of membrane proteins (Sundberg et al. 2008). These proteins transport a big range of substances over the cell membrane. Some of these substances are sugar, amino acids, nucleotides and inorganic ions (Hediger et al. 2004). There are many different families of solute carriers identified today and one of the interesting amino acid transporting solute carrier family is the SLC38 family. One of the major physiological role of SLC38 family transporters is to transport glutamine from astrocytes to neurons (Mackenzie and Erickson 2004).

The SLC38 family consists of eleven proteins and five of them (SLC38A7-SLC38A11) have been identified in our group (Sundberg et al. 2008). The solute carrier family-38 is sodium-coupled neutral amino acid transporters (SNATs). Five of these proteins SLC38A1-SLC38A5 are well-studied; whereas, the rest of the six are still orphans. Amino acid transporter can be classified to system A and system N amino acid transporters depending on their functional properties and patterns of substrate recognition. The six amino acid transporters SLC38A6 to SLC38A11 are not yet classified according to the N/A system. The orphan transporters SLC38A7-SLC38A11 have relatively low sequence similarities in comparison with the characterized transporters. The expression pattern of the orphan transporters is unknown and is of importance for our understanding of functions for these transporters (Mackenzie and Erickson 2004, Hagglund et al. 2011). SLC38A1 and SLC38A2 are found to be expressed only in neurons. There is a lack of the protein SLC38A3 in the neurons in contrast to SLC38A1 and SLC38A2. SLC38A3 can be found in the brain, more specifically in astrocytes. SLC38A4 is expressed in liver and in placenta cells. The last protein in the SLC38 family whose expression pattern is well characterized is the SLC38A5. This transporter is expressed in brain, liver, kidney, lung, colon, small intestine, stomach, and spleen.
SLC38A6 is an orphan transporter with not so well characterized expression patterns. However, expressed sequence tags have been found in placenta, spleen, muscle, brain and lungs (Sundberg et al. 2008). The transporter was detected in all tissue of mouse, with high levels in olfactory bulb and hypothalamus (Hagglund, unpublished).

In this study we used in situ proximity ligation assay (PLA) to identify interaction patterns of SLC38A6. We have shown that SLC38A6 interact with various neuronal proteins to a different extent. SLC38A6 was found to interact with vesicular proteins, membrane proteins and ubiquitously expressed proteins in the synapses. The specificity and purity for SLC38A6 was attempted to verify by western blot analysis. Immunocytochemistry was done on the proteins to verify their expression patterns in the cells and to optimize the concentration of antibodies for PLA.

Materials and Methods

Adult male C57B16/J was sacarified by decapitation. The brain was fixed in zinc-formalin for 18-24 h at 40 ° C and then dehydrated and paraffin infused. Finally, 7 µm sections were cut by using Microm 355S STS cool cut microtome (Hagglund et al. 2011).

Cell culture: For this study, immortalized cell line N25/2 (Cellutions Biosystem) was used. Cell medium was consisted of 500 ml Dulbecco's Modified Eagle Medium (DMEM) [+] 4.5g/L D-Glucose, [+] L-Glutamine, [+] Pyruvate with added 50 ml Fetal bovin serum (FBS), 5 ml Pen-Strep and 5 ml amphotericin B. Cell cultures was incubated at 37 °C with 5% CO_2.

Fluorescent immunocytochemistry on N25/2 cells: N25/2 cells were grown on 10µg/ml Poly-L-Lycine-coated slides in the medium described above. When optimal cell density was obtained (approximately 0.5 million cells/cm^2), the slides were rinsed with 1X PBS (Sodium perborate) and 4% Paraformaldehyd (PFA) was added for 15 minutes to fix the cells. Additional washes with PBS were performed before the slides were blocked in supermix (Tris-buffered saline, 0.25% gelatin, 0.5% Triton X-100) for 1 hour. The cells were incubated with primary antibodies (concentrations are summarized in table 1) anti-Synaptotagmin I raised in rabbit (Abcam) and anti-Synaptotagmin I raised in mouse (Millipore) overnight at 4 °C. The slides were rinsed in PBS 3x10 min. Secondary antibody was added Alexa Fluor ® 594 Goat Anti-Rabbit IgG and Alexa Fluor ® 594 Goat Anti-mouse IgG (Life technologies) diluted 400 times in supermix and the slides were incubated for 2h at room temperature. After washing with PBS 3x10 min the slides were incubated with DAPI (4, 6 – Diamidino-2-phenylindole dihydrichloride (Sigma-Aldrich, USA)) diluted 2500 times in PBS for 10 min. The slides were mounted in DTG. Images were taken by a Zeis Axioplan 2 and analyzed with AxioVision Rel. 4.8 software (Zeiss, Germany).

Fluorescent double immunocytochemistry (ICC): For the double ICC, the procedure was as described above except that WGA (2µg/ml in PBS) was added after the first washing with PBS before the addition of PFA and followed with additional washing.

In situ proximity ligation assay (PLA): The NS25/2 cell lines was cultured on microscope slides. Cells were fixed in 4 % PFA (Sigma-Aldrich, USA) for 15 min. The in situ Duolink II fluorescence kit (orange detection reagent, Olink Biosciences, Sweden) was used for PLA procedure according to the manufacturer's instructions (Fredriksson et al. 2002, Soderberg et al. 2006, Jarvius et al. 2007). The primary antibodies that were used are summarized in table 2.

Image analysis: For the PLA and Immunocytochemistry pictures were taken on a Zeis Axioplan 2 and analyzed with AxioVision Rel. 4.8 software (Zeiss, Germany). Z-stack images from different sites of the slides were taken and PLA signals were counted with Duolink ImageTool (Olink) software. The average signal per cell was calculated and presented with 95% confident interval. The result was compiled and analyzed in GraphPad prism 5 software.

Western Blot: For two proteins Synaptotagmin (mouse) and Synaptophysin (mouse), we did western blot procedure on brain tissue. Gel electrophoresis was run on a Mini-Protean TGX gel (4-10%, Bio-Rad) in running buffer (0.1%SDS, 0.025 Tris base, 0.192 M glycine) on protein sample. PageRuler prestained protein ladder (Fermentas, Canada) was run together with the protein sample. The proteins were then transferred to Immobilon-P PVDF membrane (Millipore) in transfer buffer (0.025 M Tris base, 0.192 M glycine, 20% methanol). The membrane was blocked in Blocking buffer (5% nonfat dry milk in 1.5 M NaCl, 0.1M Tris, 0.05% Tween 20, pH 8.0) for 1 hour. After the membrane was cut into small pieces they were incubated with the primary antibodies over night (4° C). After incubation over night the membrane pieces was washed with water and later incubated with secondary antibodies for 1 hour. The secondary antibody that was used was goat anti-mouse horseradish peroxidase-conjugated secondary antibody diluted 10000 times in blocking buffer. For the blot development Luminal/enhancer (immune-Star HRP) was mixed with peroxidase buffer solution (BioRad) in 1:1 ratio. The mixture was added to the membrane and placed in a cassette for 1 min. The development was done on a high performance chemiluminescence film (GE Healthcare).

Results

WGA staining for determination of the average cell size: WGA staining was used on our cells to determine the average size of N25/2 cells. The size was decided in pixels and fed into the Duolink ImageTool software as input data. It was of big importance to determine the cell size for the signal per cell calculations. The result of the ICC with WGA staining is shown in figure 1.

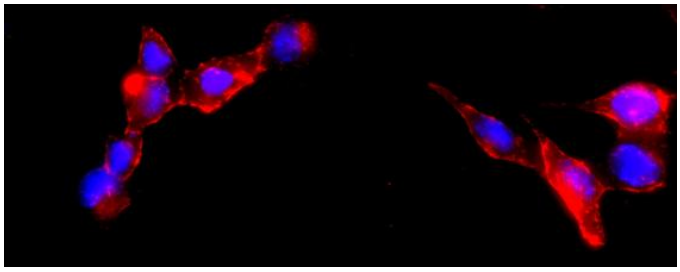


Figure 1:Fluorescence ICC with DAPI and WGA on N25/2 cells-
ICC on N25/2 cells to determine the cell nucleus and cell size in pixels. The red is WGA showing the cell membrane and the blue is DAPI showing the nucleus. The image is 40x magnified and taken on a Zeis AxioPlan 2 fluorescence microscope and analyzed with AxioVision Rel. 4.8 software.

Immunocytochemistry: We determined the best dilution of the commercial Synaptotagmin (Milipore; Abcam) to 1:100 using Immunocytochemistry. For other proteins used in this study, optimizations have been done earlier by our group and are summarized in table 1 (unpublished data)
The same concentrations of the antibodies were consistently used throughout this study for valid comparison.

Table 1. Fluorescence-ICC concentrations

1° antibody	Concentration	2° antibody	Concentration
Rabbit-anti-Synaptotagmin I (Abcam, UK)	1:100	Alexa flour 594 goat-anti-rabbit IgG (H+L) (Invitrogen, USA)	1:400
Mouse-anti-Synaptotagmin I (Millipore, Sweden)	1:100	Alexa flour 594 goat-anti-mouse IgG (H+L) (Invitrogen, USA)	1:400

Optimal concentrations for the PLA assay determined by ICC.

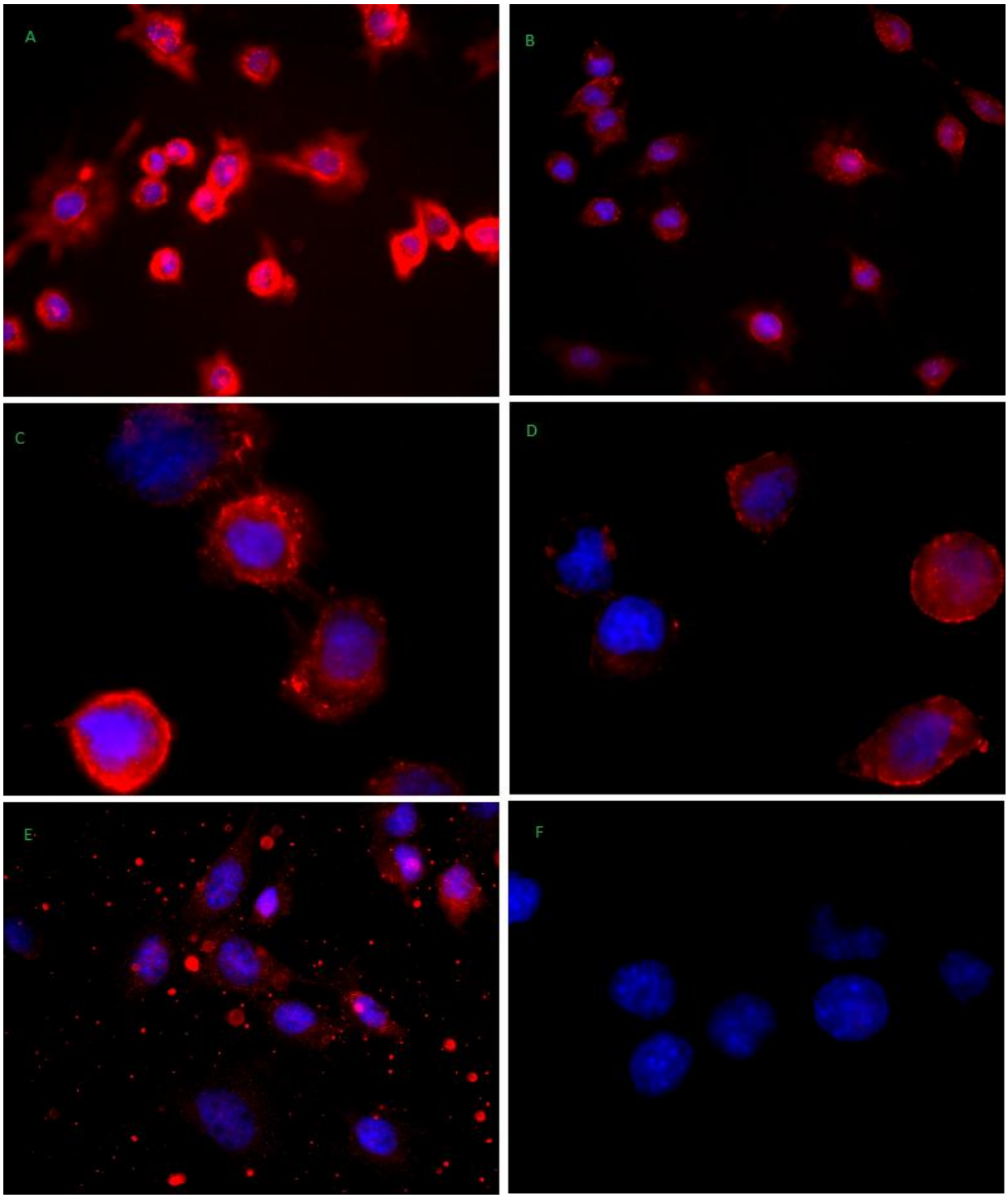


Figure 2. Fluorescence-ICC images. (A) Cells incubated in rabbit-Synaptotagmin I diluted 1:50 and DAPI; picture in 40x, (B) rabbit-Synaptotagmin I diluted 1:100 and DAPI; picture in 40x, (C) rabbit-Synaptotagmin I diluted 1:50 and DAPI; picture in 100x, (D) rabbit-synaptotagmin I diluted 1:100 and DAPI; picture in 100x, (E) mouse-Synaptotagmin I diluted 1:100 and DAPI; picture in 40x, (F) negative control with no primary antibody with DAPI; picture in 100x.

SLC38A6 interacts with various proteins in the cell: Proximity ligation assay technique showed interaction between our target protein and the other known proteins. PLA performed on tissue sections indicated interaction between SLC38A6 and synaptophysin, however quantitative measurements couldn't be done on tissue sections due to the fact that the cells in tissue section are merged and cannot be counted as individual cells. No protein interactions were seen in the negative control (figure 3B, H). For quantitative measurements, PLA was performed on cell cultures where individual cells could be visualized separately. The degree of interaction between the proteins and SLC38A6 is summarized in figure 4.

Table 2 - SLC38A6 concentrations for PLA.

Type	Protein 1	Species	Dilution	Protein 2	Species	Dilution
Tissue	SLC38A6	Rabbit	1:200	Synaptophysin	Mouse	1:250
Tissue	SLC38A6	Rabbit	1:200	Synaptophysin	Mouse	1:250
Cell	SLC38A6	Rabbit	1:200	Synaptophysin	Mouse	1:250
Cell	SLC38A6	Rabbit	1:200	Snap-25	Mouse	1:500
Cell	SLC38A6	Rabbit	1:200	Synaptotagmin	Mouse	1:100
Cell	Synaptotagmin	Rabbit	1:100	Synaptophysin	Mouse	1:250
Cell	Synaptotagmin	Rabbit	1:100	Snap-25	Mouse	1:500

Concentrations and combinations for the different proteins used in the PLA assay experiment. The PLA was performed on paraffin brain tissue sections and on N25/2 cell lines. The two proteins used in PLA had to be raised in different species represented in the column "Specie" for respective proteins.

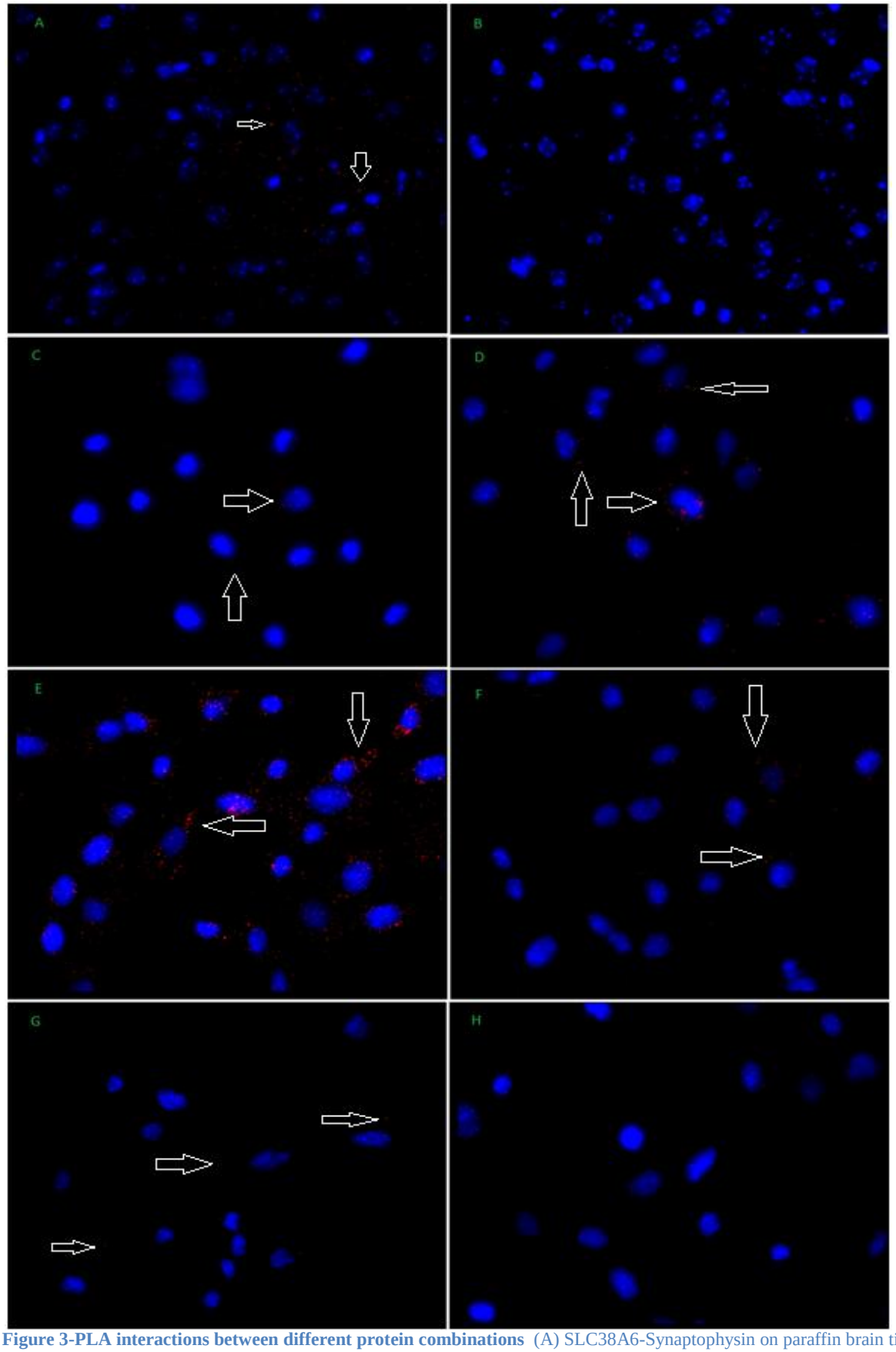


Figure 3-PLA interactions between different protein combinations (A) SLC38A6-Synaptophysin on paraffin brain tissue sections, (B) control without primary antibodies on paraffin brain tissue sections, (C) SLC38A6-Synaptophysin on cells, (D) SLC38A6-Snap 25 on cells, (E) SLC38A6-Synaptotagmin on cells, (F) Synaptotagmin -Snap 25 on cells, (G) Synaptotagmin - Synaptophysin on cells, (H) control without primary antibodies on cells.

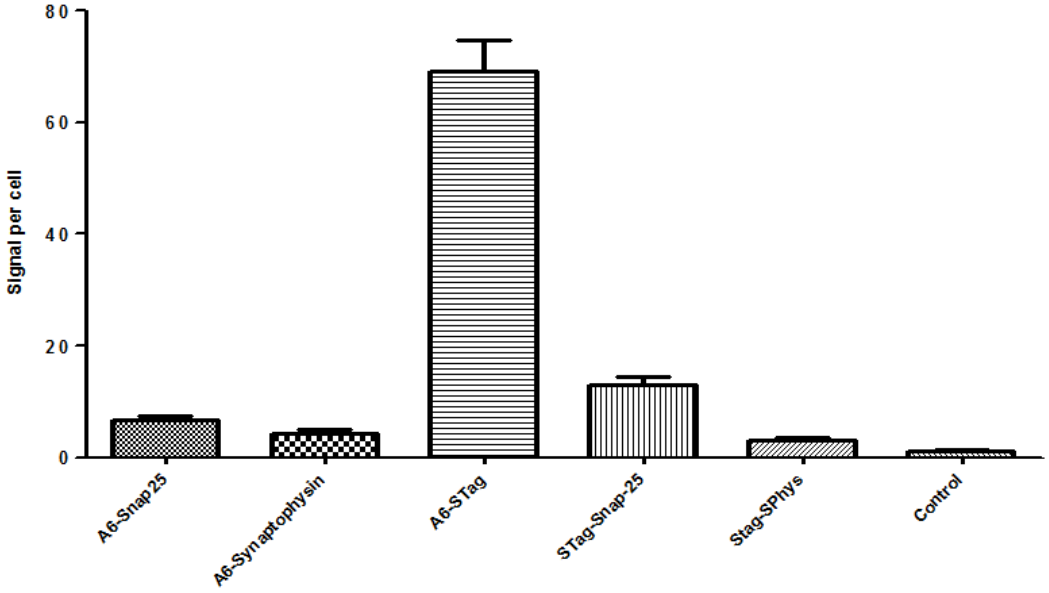


Figure 4 - *PLA performed on NS25/2 cell lines.* *PLA signals per cell presented as means with 95% CI- A bar chart diagram showing the mean value for signals per cell with error bars showing the 95% confident interval. The first bar shows the signals per cell for the PLA signal result between SLC38A6 and Snap-25 followed by SLC38A6-Synaptophysin, SLC38A6-Synaptotagmin, Synaptotagmin-SNAP-25, Synaptotagmin-Synaptophysin and the control (no primary antibodies added) respectively. For more details see table 2. Student T-test was performed between the tested proteins and the control with a value $p<0.005$ indicating significantly different results. The bar chart diagram and statistical analysis were done in GraphPad Prism 5 software.*

Western blot verified specificity for two of the tested proteins: With western blot, the specificities of the monoclonal antibodies mouse Anti-Synaptotagmin (Millipore) and mouse Anti-Synaptophysin (Abcam) were verified. The 65 kDa Anti-Synaptotagmin protein and the 34 kDa Anti-Synaptophysin were detected within the expected range in the western blot.

Discussion

Recent studies done by our group show that SLC38A6 is widely expressed in the brain with high expression in hypothalamus and olfactory bulb (Sundberg et al. 2008). Theoretically, it appears that, SLC38A6 is a membrane protein involved in the transport of various amino acids over the cell membrane. We wanted to experimentally show the localization and interaction pattern of SLC38A6 in relation with other relevant synaptic proteins. This would strengthen our hypothesis of SLC38A6, being a membrane protein involved in the transport of various amino acids over the cell membrane. Hence, we studied the interaction pattern for various proteins with SLC38A6 and presented the result as signals per cell. The number of signals reflects the extent of interaction between the proteins. For determination of the cell size of our cell line N25/2 we did double IHC with DAPI (stained the cell nucleus) and WGA (stained the cell cytoplasm). These measurements enabled us to determine shape and more importantly, size of the cells (figure 1). The average cell size was determined in pixels and can be used further for the signal per cell measurements in the same cell line under same conditions.

Our result in this study shows that, SLC38A6 interacts more with the vesicular protein Synaptotagmin compared to Snap-25 and Synaptophysin (figure 4). The obvious explanation for this can be that SLC38A6 is localized at the vesicles instead of the membrane. We have no direct proof of its localization otherwise, but considering quite high possibility of SLC38A6 being involved in amino acid transportation, there is a bigger chance of SLC38A6 being localized at the membrane rather than on the vesicles. It is also possible that even though SLC38A6 is present on the membrane, they are interacting both with membrane proteins and vesicular proteins that are present only on the vesicles near the membrane (see figure 5).We have, however, no account for the extent of expression of these individual proteins in the cells. Therefore, PLA would show more signals for a protein with higher expression level (for example, vesicular proteins in this case) compared to one that is expressed much less in each cell (for example, membrane proteins). In this scenario, the PLA signals per cell will be much higher in interaction between SLC38A6 and Synaptotagmin compared to that of SLC38A6 and Snap25. To support our speculations, PLA between Synaptotagmin and Snap-25 explicitly shows countable proximity between membrane proteins and vesicular proteins (See Figure 4). We will run PLA between same proteins raised in different species. This might give us an account of expression of these proteins. We propose a model on the basis of these speculations (See Figure 5). We still have to run several control experiments to prove this hypothesis concerning the localization.

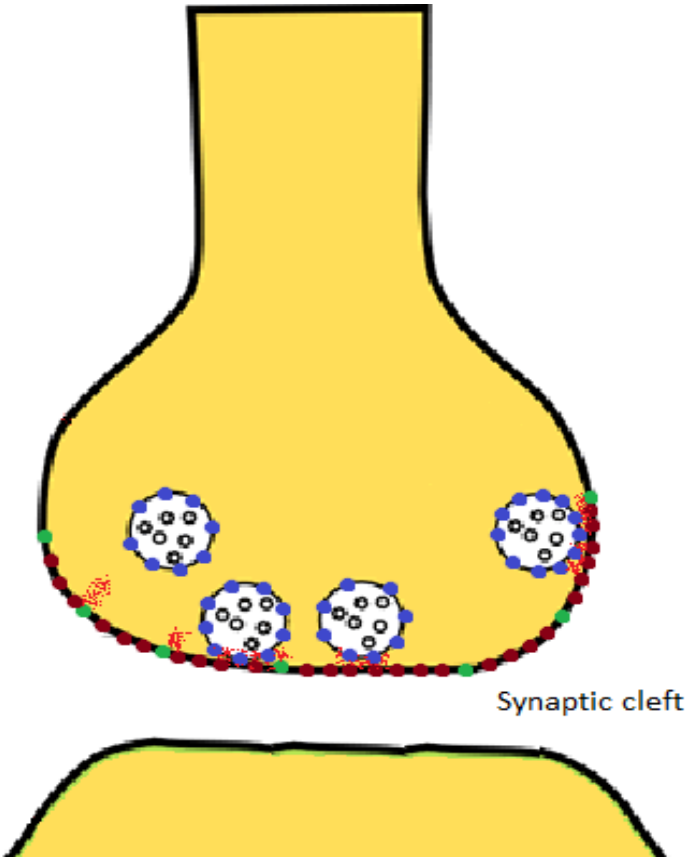


Figure 5. Model of current hypothesis. *The figure illustrates synaptic vesicles (rings) at a synapse of a neuronal cell. The green marking indicates membrane proteins, blue markings indicates synaptic vesicular proteins, brown markings indicate SLC38A6 and the red dots demonstrate PLA signals.*

References

Fredriksson, S., M. Gullberg, J. Jarvius, C. Olsson, K. Pietras, S. M. Gustafsdottir, A. Ostman & U. Landegren **(2002)** Protein detection using proximity-dependent DNA ligation assays. *Nat Biotechnol,* 20, 473-7.

Hagglund, M. G., S. Sreedharan, V. C. Nilsson, J. H. Shaik, I. M. Almkvist, S. Backlin, O. Wrange & R. Fredriksson **(2011)** Identification of SLC38A7 (SNAT7) protein as a glutamine transporter expressed in neurons. *J Biol Chem,* 286, 20500-11.

Hediger, M. A., M. F. Romero, J. B. Peng, A. Rolfs, H. Takanaga & E. A. Bruford **(2004)** The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch,* 447, 465-8.

Jarvius, M., J. Paulsson, I. Weibrecht, K. J. Leuchowius, A. C. Andersson, C. Wahlby, M. Gullberg, J. Botling, T. Sjoblom, B. Markova, A. Ostman, U. Landegren & O. Soderberg **(2007)** In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. *Mol Cell Proteomics,* 6, 1500-9.

Mackenzie, B. & J. D. Erickson **(2004)** Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch,* 447, 784-95.

Soderberg, O., M. Gullberg, M. Jarvius, K. Ridderstrale, K. J. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L. G. Larsson & U. Landegren **(2006)** Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods,* 3, 995-1000.

Sundberg, B. E., E. Waag, J. A. Jacobsson, O. Stephansson, J. Rumaks, S. Svirskis, J. Alsio, E. Roman, T. Ebendal, V. Klusa & R. Fredriksson **(2008)** The evolutionary history and tissue mapping of amino acid transporters belonging to solute carrier families SLC32, SLC36, and SLC38. *J Mol Neurosci,* 35, 179-93.

Wu, C. C., M. J. MacCoss, K. E. Howell & J. R. Yates, 3rd **(2003)** A method for the comprehensive proteomic analysis of membrane proteins. *Nat Biotechnol,* 21, 532-8.