An *in vitro* investigation of NECAB2 membrane interactome

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**NECAB2** is a Ca\(^{2+}\)-binding protein highly expressed in brain, known to interact with G protein-coupled receptors and altering receptor behavior and downstream signaling. Here we investigate potential NECAB2 interactors *in vitro* using NECAB2 transfected HEK293 cells and co-immunoprecipitation and mass spectrometry approaches. NECAB2 containing protein complexes were isolated and analyzed using mass spectrometry. MARCKS, known to be involved in dendritic spine formation, was identified as a NECAB2 interacting protein. This interaction might explain the morphological changes and formation of neurite-like structures observed in NECAB2 transfected HEK293 cells. We have established an *in vitro* model system to study the NECAB2 interactome. However, these findings are still preliminary and additional experiments are needed to validate these results.

**Keywords:** Ca\(^{2+}\)-binding protein; co-immunoprecipitation; membrane protein extraction; protein-protein interaction

**Abbreviations:** 5-HT, serotonin; A2A, adenosine 2A; CaM, calmodulin; coomassie staining (CS); ED, effector domain; GPCR, G protein-coupled receptor; ICC, immunocytochemistry; IgG, immunoglobulin G; IP, immunoprecipitation; MARCKS, myristoylated alanine-rich C-kinase substrate; mGlu5b, metabotropic glutamate type 5; MS, mass spectrometry; NECAB, N-terminal calcium binding protein; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C; SDC, sodium deoxycholate; TMED10, transmembrane emp24 domain-containing protein 10; WB, western blot

**Introduction**

Ca\(^{2+}\) is important for the release of neurotransmitters and neural signaling, and there are numerous Ca\(^{2+}\)-binding proteins, calmodulin (CaM) being one of the most well known (Burgoyne et al. 2004). The N-terminal calcium binding protein 2 (NECAB2) is a 43.2 kDa protein that belongs to a recently identified Ca\(^{2+}\)-binding protein family containing three proteins, that share regions of homology: a unique EF-hand containing domain at the N-terminus containing a single Ca\(^{2+}\)-binding site, a NECAB homology region with a coiled-coiled domain that is highly conserved, and a DUF176/ABM motif at the C-terminal. In the rat (Sugita et al. 2002) and human (http://www.proteinatlas.org) NECAB1 and NECAB2 are highly expressed in brain whereas NECAB3 is highly expressed in many tissues including brain and muscle. Detailed mapping of NECAB2 distribution in the mouse brain revealed high expression of NECAB2 in cortex, CA2 area of hippocampus and the dorsal raphe nuclei where it also co-localizes with serotonin (5-hydroxytryptamine, 5-HT) (Figure 1). NECAB2 has a strong association to the plasma membrane of cells (data not shown) and is known to interact with the C-terminal domain of G protein-coupled receptors (GPCRs), including the adenosine 2A (A\(_{2A}\)) receptor (Canela et al. 2007) and metabotropic glutamate type 5 (mGlu\(_{5b}\)) receptor (Canela et al. 2009). An increase in internal Ca\(^{2+}\) concentration promotes dissociation from these receptors. Cells co-expressed with NECAB2 and the A\(_{2A}\) receptor has reduced receptor cell surface expression, by promoting intracellular retention of the receptor, but at the same time an increased response of ERK1/2 phosphorylation after agonist stimulation of the receptor (Canela et al. 2007). This could mean that NECAB2 regulates receptor cell surface expression and downstream signaling efficacy. Also, when co-expressed with the mGlu\(_{5b}\) receptor an accumulation of
An *in vitro* investigation of NECAB2 membrane interactome

inositol phosphates was observed after agonist stimulation as well as an increase in phosphorylation of ERK1/2 (Canela et al. 2009).

5-HT is a monomeric neurotransmitter that plays an important role in physiological systems and behavior, and is involved in psychiatric disorders such as anxiety, depression and schizophrenia. Most of forebrain 5-HT is derived from 5-HT-containing neurons in dorsal and median raphe nuclei (Lucki 1998). The 5-HT<sub>1A</sub> receptor is highly expressed in 5-HT neurons in soma and dendrites of the dorsal raphe nuclei, regulating 5-HT cell firing and 5-HT release in the forebrain via an inhibitory feedback loop (Barnes & Sharp 1999). The 5-HT<sub>2C</sub> receptor has also been implicated in 5-HT inhibitory feedback of neuronal cell firing and 5-HT release, but on the postsynaptic site (Sharp et al. 2007). Understanding the mechanisms that fine-tune 5-HT receptor activity could help designing novel therapies treating disorders such as anxiety and depression. One way could be to target not the receptor itself but proteins that regulate receptor trafficking and signaling.

Since NECAB2 is expressed in 5-HT-neurons in the dorsal raphe nuclei (Figure 1b) and has been shown to interact with GPCRs, one potential interactor is the 5-HT<sub>1A</sub> receptor. The 5-HT<sub>2C</sub> receptor was also chosen for investigation because of its postsynaptic regulation in contrary to the 5-HT<sub>1A</sub> receptor’s presynaptic regulation. To investigate this potential interaction, a pull-down strategy optimized for capturing protein-protein interactions at the plasma membrane had to be established. We co-transfected HEK293 cells with NECAB2 and one of the 5-HT receptors. Pull-down efficiency and NECAB2 interacting partners were determined using western blot (WB) and mass spectrometry (MS).

![Figure 1. Distribution of NECAB2 in mouse brain. Brain slices were processed by immunofluorescence using antibodies against NECAB2 (green) and 5-HT (red) as well as DAPI for the nucleus (blue). (A) Localization of NECAB2 and 5-HT in the dorsal raphe nuclei, (B) and co-localization in cells, (C) NECAB2 is shown to be highly expressed in cortex, the dorsal raphe nuclei and (D) CA2 area of the hippocampus. Pictures were provided by Tissue Profiling, Karolinska Institute.](image-url)
**Results**

**Plasmid preparation and amplification**

The NECAB2 encoding gene was fused to a Myc-DDK tag at the C-terminal, giving the protein a total weight of 45.4 kDa. It is expressed under a CMV promoter (Figure 2). The NECAB2 and 5-HT<sub>2C</sub> plasmids were amplified in transformed *E. coli*, purified and analyzed by light spectrometry, and highly pure plasmid solutions with an optical density of ≥1.8 were used for transfection.

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Transfection of NECAB2 was confirmed by WB. Cell lysate from transiently transfected HEK293 cells had two immunoreactive bands (Figure 3a), ~45 kDa and ~43 kDa respectively, corresponding to NECAB2 as shown by earlier

**HEK293 cell transfection and optimization**

Figure 3. Plasmid map of the NECAB2 plasmid used for transfection of the HEK293 cells. A Myc-DDK tag is fused to the C-terminal of NECAB2 that is expressed under a CMV promoter.

Figure 2. (A) HEK293 cells were transiently transfected with NECAB2. Western blot from cell lysate of transiently transfected (N2), non-transfected control cells (Ctrl) and mouse cortex (CTX), analyzed using the HPA014414 anti-NECAB2 antibody and detected as described in experimental methods. (B) Immunocytochemistry image of fixed, NECAB2 transiently transfected HEK293 cells, showing the cell nucleus (blue) and expression of NECAB2 (red). (A) HEK293 cells were transiently transfected with the 5-HT<sub>2C</sub> receptor for validation of the anti-5-HT<sub>2C</sub> antibody. Western blot from cell lysate of transiently transfected (2C), non-transfected control cells (Ctrl) and mouse cortex (CTX), analyzed using the primary anti-5-HT<sub>2C</sub> antibody and detected as described in experimental methods.
An *in vitro* investigation of NECAB2 membrane interactome

reports (Canela et al. 2007) and in-house data (data not shown). NECAB2 was visualized using immunocytochemistry (ICC), and transfection rate was calculated by dividing number of transfected cells with total number of counted cell nuclei using ImageJ (NIH, US). One DAPI stained nucleus was counted as one cell and was compared to the immunostaining of NECAB2 where overlap of one nucleus with the immunostaining was counted as one transfected cell (Figure 3b, Table 1). First the plasmid to transfection reagent ratio to use was determined using 1 µg of plasmid per 3×10^5 of seeded cells and a plasmid to transfection reagent ratio of 1:2-1:6. Secondly the amount of plasmid was optimized using 0.5, 1.0 or 1.5 µg of plasmid with a plasmid to transfection reagent ratio of either 1:4 or 1:5. Optimal transfection rate was found when using 1 µg of plasmid per 3×10^5 of seeded cells with a plasmid to transfection reagent ratio of 1:4 with approximately 25% of the cells expressing NECAB2.

**Cell morphology change**

Some of the transiently transfected HEK293 cells had a distinct morphological change (Figure 4). NECAB2 immunoreactivity is visualized in red and yellow, and can be seen all along the outgrowths, and also something that looks like deposits or accumulations of NECAB2. Parallels can be drawn to the shape of neurites. This indicates a morphology change induced by the introduction of NECAB2.

**Antibody validation**

The anti-NECAB2 antibodies had already been validated by the lab prior to this project (data not shown). The anti-5-HT_1A_ antibody never arrived and the anti-5-HT_2C_ antibody specificity was validated by WB on HEK293 cells transiently transfected with the 5-HT_2C_ receptor and on mouse cortex (Figure 3c), as well as ICC on transiently transfected HEK293 cells (data not shown). Unfortunately it failed revealing an immunoreactive band around ~50 kDa in WB or any positive immunofluorescence staining in the ICC.

**NECAB2 protein complex pull-down by co-immunoprecipitation**

To identify NECAB2 membrane interactome a pull-down of NECAB2 protein complexes using co-immunoprecipitation (co-IP) was used followed by MS analysis. To determine optimal conditions and antibody to use for the co-IP both the anti-NECAB2 antibodies and an anti-FLAG antibody were tested (Figure 5a). The HPA013998 (lane 1) and the anti-FLAG (lane 4) antibody were both compatible with IP as
they showed two strong immunoreactive bands as compared to the immunoglobulin G (IgG) control (lane 3 for HPA013998 and lane 5 for anti-FLAG), but not the HPA014414 (lane 2) as it had two immunoreactive bands with approximately the same intensity as the IgG control (lane 3).

To see if NECAB2 also pulled down other proteins in complex, a coomassie staining (CS) on a SDS-PAGE of a co-IP from crude cell lysate from transfected HEK293 cells, followed by WB was performed (Figure 5b). The NECAB2 and IgG control co-IP showed numerous protein bands (lane 1 and 2) and the WB confirmed the presence of NECAB2 in the transfected sample (lane 3) and not in the control sample (lane 4). A comparison between the relative intensities of the CS bands (Figure 5c) showed that the NECAB2 sample had more proteins in the weight range of ~100-40 kDa, indicating that NECAB2 protein complexes were pulled down rather than a single protein.

To optimize the detergent for the co-IP and to make it MS compatible, the used detergent has to be compatible with the column but also has to be strong enough to be able to lyse the cell membrane effectively to release the membrane bound proteins bound. CHAPS (a mild non-ionic detergent) was first tried for cell lysing (Figure 6a) in combination with Ca\(^{2+}\) enriched and depleted (addition of EDTA) membrane samples. It worked well for extraction of NECAB2 in crude lysate (lane 1) but probably only cytosolic NECAB2 was extracted, as the extraction from both the membrane fractions (lane 3 and 5) were negative for NECAB2 when compared to controls (lane 4 and 6). To see if sodium deoxycholate (SDC) (a mild non-ionic detergent) was strong enough for membrane protein extraction it was compared to Triton X-100 (a strong ionic detergent) in a CS (Figure

Figure 5. Immunoprecipitation of NECAB2 transiently transfected HEK293 cells, analyzed using the HPA014414 anti-NECAB2 antibody and visualized as described in experimental methods. (A) Western blot analysis to determine which antibody to use for the pull-down. HPA013998 (1) and HPA014414 (2) antibodies were tried and compared to a IgG control (3), as well as the anti-FLAG antibody and a IgG control (5) but in a separate experiment. (B) Coomassie staining of a co-immunoprecipitation followed by a western blot to determine if there are protein differences between NECAB2 (N2) and IgG control (IgG) pull-downs. (C) Lane intensity comparison between NECAB2 (dark green) and IgG control (light green) pull-down.
An in vitro investigation of NECAB2 membrane interactome

6b) on crude lysate from non-transfected HEK293 cells. As we encountered difficulties extracting membrane associated NECAB2 and bound interactors, a formaldehyde cross-linking strategy was implemented, increasing the chance of finding NECAB2 interactors. This showed that SDC freed protein from the cross-linked membrane fraction (lane 6) almost as efficient as Triton X-100 (lane 5). There are also intense bands at the top of lane 5 and 6 that could be large cross-linked protein complexes, partially explaining the weaker intensity of the cross-linked lanes compared to the non cross-linked lanes (lane 3 and 4). Before MS analysis of the co-IP samples we confirmed that NECAB2 was present in the NECAB2 pull-down and not in the IgG control. For this, a CS on the SDS-PAGE of the co-IP followed by a WB was made (Figure 7a). Lane 1 shows a band at ~45 kDa that corresponds to NECAB2 as well as two other bands at ~25 kDa and ~17 kDa that could correspond to protein interacting with NECAB2, as these bands did not seem to be present in the IgG control sample (lane 2). To confirm the presence of NECAB2 in the co-IP sample a WB was run (Figure 7b). It showed an immunoreactive band at ~33 kDa which could correspond to a partially fragmented, a splice variant or a not fully denatured form (due to cross-linking) of NECAB2. Since the HPA014414 antibody never has shown any cross reactivity in WBs in this project or in other experiments performed in the lab (data not shown), it was assumed the sample contained NECAB2.

Mass spectrometry analysis of co-immunoprecipitation samples

NECAB2 and control IgG samples from the co-IP were analyzed by MALDI-TOF MS/MS by Janne Lethio’s (Karolinska Institutet) group, and the NECAB2 sample produced a total of 82 unique proteins when compared to the control sample. These include myristoylated alanine-rich C-kinase substrate (MARCKS) and transmembrane emp24 domain-containing protein 10 (TMED10). MARCKS is interesting because it is involved in brain development, endo-, exo-, and phago-cytosis as well as neurosecretion (Arbuzova et al. 2002). TMED10 in turn, is associated with certain

Figure 6. Detergent optimization for cell lysing of NECAB2 transiently transfected HEK293 cells. (A) Western blot using the HPA014414 anti-NECAB2 antibody as described in experimental methods. CHAPS was used for cell lysing (see experimental methods for details) of crude (Crude) or membrane (Membrane) fractions, which was used for immunoprecipitation of NECAB2 (N2) or IgG control (IgG). Also, for the membrane fraction, either Ca\(^2+\) enriched (Ca\(^2+)\) or depleting (EDTA) detergents were used. (B) Coomassie staining of SDS-PAGE analyzed crude (Crude) or membrane (Membrane) fractions for comparison between the cell lysing capabilities of the detergents Triton X-100 (TX) and sodium deoxycholate (SDC). Also, proteins were either cross-linked (CL) or not cross-linked (Non-CL) before cell lysing.
protein kinase Cs (PKCs) and is also involved in vesicular protein trafficking (Blum & Lepier 2008; Wang et al. 2011).

Discussion

In this project we for the first time explored and identified potential NECAB2 protein interactors. Because of the limited project time, several results need to be confirmed and protocols need further adjustment. Nevertheless, we were able to identify potential NECAB2 interactors as well as develop or further tweak established methods for extracting and identification of membrane protein complexes.

It was unfortunate that the anti-5-HT$_{1A}$ antibody never arrived and that the anti-5-HT$_{2c}$ antibody was too unspecific for use, but instead more time could be put into optimizing the co-IP so that it could be used for MS analysis. Regarding the optimization of the in vitro system, a high transfection rate was not needed but rather enough transfected cells to be able to conduct the experiments and therefor, the rather low transfection rate of approximately 25% could be accepted. The development of the co-IP protocol was successful, but further tweaking of the co-IP needs to be made to reach higher protein recovery and confirm reproducibility, and also make it viable in vivo. One thing that could be tried is to reduce the high formaldehyde concentration of 11% to ≤1% and try different exposure times, and by doing so hopefully increase protein recovery (Sutherland et al. 2008).

What has been done in this project needs to be validated and tested in a more systematic manner. But, if it is assumed that these findings are true it could be speculated in how these proteins interact and the consequences of these interactions.

MARCKS is expressed in many tissues of rat, especially brain, and other mammals including human (Albert et al. 1986), and has been implicated in secretion, cell motility, transformation and neurite outgrowth, all processes that needs rearrangement of actin at the plasma membrane (Aderem 1992; Laux et al. 2000). MARCKS binds membranes with a myristoylated domain located at the N-terminal that inserts into the hydrophobic bilayer, and a basic effector domain (ED) interacting with acidic lipids via electrostatic interactions (Mclaughlin & Aderem 1995), and furthermore, phosphorylation or binding of Ca$^{2+}$-CaM to the ED, were one inhibits the other, dissociates MARCKS from the membrane (Arbuzova et al. 2002). MARCKS interacts directly with phosphatidylinositol 4,5-bisphosphate (PIP$_2$) creating microdomains at the membrane as well as inhibiting phospholipase C (PLC) dependent hydrolysis of PIP$_2$, and it is proposed that upon activation of PKC or Ca$^{2+}$-CaM these local clusters of PIP$_2$ are released, promoting actin filament assembly resulting in i.e. growth of axons (Glaser et al. 1996; Laux et al. 2000). Also, phosphorylation of MARCKS by PKC is inhibited when MARCKS is bound to PIP$_2$ (Seki et al. 1996).

Our results show that introduction of NECAB2 changes the morphology of HEK293 cells and that NECAB2 interacts directly or indirectly with MARCKS. MARCKS is necessary for brain development (Stumpo et al. 1995) and this interaction with NECAB2 could.

![Figure 7. Validation of protein difference and NECAB2 presence in co-immunoprecipitated NECAB2 (N2) and IgG control (IgG) samples using sodium deoxycholate as detergent on NECAB2 transiently transfected HEK293 cells. (A) Coomassie staining (lane 1 and 2) of samples and (B) western blot, using the HPA014414 anti-NECAB2 antibody as described in experimental methods.](image-url)
be relevant for this development and might also underpin the morphological changes found in NECAB2 transfected HEK293 cells. A possible molecular mechanism for this interaction is that in resting condition, NECAB2 is bound to GPCRs in close proximity to PIP$_2$ microdomains sequestered by MARCKS and upon activation of the receptor NECAB2 binds MARCKS, exposing PIP$_2$ for hydrolysis by PLC. Release of IP$_3$ increases intracellular Ca$^{2+}$ concentrations which bind NECAB2 that then dissociates from the GPCR and MARCKS, and the simultaneous increase in DAG activates PKC that now phosphorylates MARCKS, inhibiting re-binding to the membrane and results in a local actin filament assembly, promoting secretion or outgrowth (Figure 8).

It has been shown that MARCKS is essential in the formation and maintenance of dendritic spines and that cells treated with MARCKS RNAi have reduced spine density, spine length and spine head width (Calabrese & Halpain 2005). Inhibition of MARCKS should inhibit the dendritic morphological change induced by NECAB2, because if MARCKS is unavailable for sequestering of PIP$_2$ the local accumulation should be reduced as well as making NECAB2 unable to interact with MARCKS reducing the local hydrolysis of PIP$_2$. To test this, one could use siRNA against MARCKS in NECAB2 transfected HEK293 cells and visualize with ICC to see if it reduces morphological change in relation to control cells, NECAB2 transfected HEK293 cells and MARCKS siRNA expressing HEK293 cells. Pseudo-phosphorylated (the four serines in the ED are replaced with asparagine to mimic phosphorylation) MARCKS has reduced membrane affinity and affects cell morphology (Arbuzova et al. 2002; Calabrese & Halpain 2005) and could also be expressed in HEK293 cells as mentioned above. It could also be investigated if increased activity of PKC reduces or inhibits morphological change (i.e. use phorbol 12-myristate 13-acetate as described in Calabrese & Halpain 2005), both separately and in combination with the methods mentioned above.

It should also be investigated whether lowered or increased changes in Ca$^{2+}$ changes NECAB2 binding of MARCKS. This could be done by having NECAB2 dissolved in a physiological buffer and then incubated together with MARCKS, or do a co-IP of NECAB2 and MARCKS from transfected cells or tissue.

![Figure 8](image)

**Figure 8.** A proposed model for the interaction of NECAB2 with MARCKS. In the top panel, NECAB2 is bound to GPCRs in close proximity to MARCKS, which in turn is bound to PIP$_2$ and sequesters local domains of it. When the receptor is activated (middle panel), NECAB2 binds MARCKS and exposes PIP$_2$ for hydrolysis by phospholipases. The increase in DAG (lower panel) activates protein kinase C that phosphorylates MARCKS, inhibiting it from binding PIP$_2$, and also the increase in IP$_3$ increases Ca$^{2+}$ concentrations which releases NECAB2 from the GPCR bound state.
together with increasing concentrations of Ca$^{2+}$. It would also be interesting to see if the accumulations of NECAB2 along the outgrowths (Figure 4) is co-localized with MARCKS and/or PIP$_2$. To investigate this one could co-stain NECAB2 transfected HEK293 cells with antibodies against NECAB2, MARCKS and PIP$_2$ (i.e. as described by Laux et al. 2000) and visualize it with ICC in combination with a proximity ligation assay.

NECAB2 was also shown to interact with TMED10. This protein is expressed in all major areas of rat and human brain (Vetrivel et al. 2008) and is associated with the intermediate compartment membranes as well as the plasma membrane (Blum & Lepier 2008). TMED10 has two domains for trafficking directions. One is the KKXX motif that binds COPI proteins, which is important for retention and retrieval of KKXX-tagged proteins in the early secretory pathway, and the other is the luminal domain for targeting TMED10 to the plasma membrane. It has also been shown that masking of the KKXX motif increases translocation of TMED10 to the plasma membrane (Blum & Lepier 2008), and when located at the membrane TMED10 suppresses the activity of γ-secretase cleavage (Pardossi-Piquard et al. 2009).

Free cytosolic NECAB2 might bind TMED10, masking the KKXX motif to increase translocation to the plasma membrane. This allows for the regulation of TMED10 dependent interactions, such as the γ-secretase cleavage. To investigate this possibility, NECAB2 binding to TMED10 should be analyzed with increasing Ca$^{2+}$ concentrations. It should also be investigated in high to low intracellular Ca$^{2+}$ concentrations if NECAB2 co-localizes with TMED10 and where in the cell, using i.e. co-IP on both NECAB2 and TMED10 in co-transfected cells in combination with a proximity ligation assay.

In conclusion, this project has shown that it is possible to use co-IP on cross-linked NECAB2 transiently transfected HEK293 cells to look for potential protein interactors, which then later is validated and more thoroughly investigated using other methods, such as co-transfection in combination with co-IP and WB under different conditions. It would also be desired to apply the MS analysis on tissue to find in vivo protein interactors. This project also concludes a theory on how NECAB2 might interact with MARCKS and suggesting a regulatory role of NECAB2 at the membrane regulating fine-tuning signaling events.

**Experimental methods**

**Cell culture, plasmid preparation and transfection**

HEK293 cells were grown in Dulbecco’s modified Eagle’s medium, DMEM (Sigma Aldrich) supplemented with 100 U/ml penicillin/streptomycin and 10% fetal bovine serum (FBS), at 37 °C in an atmosphere of 5% CO$_2$. The NECAB2 plasmid was acquired from OriGene and the 5-HT$_3$ receptor plasmid from Thermo Scientific and transformed into readily E. Coli for amplification using penicillin or kanamycin for selection, and purified using PureLink™ HiPure Plasmid Miniprep Kit (Invitrogen). HEK293 cells were transiently transfected using ViaFect™ Transfection Reagent (Promega) according to manufacturers instructions and harvested 24 h after transfection unless otherwise stated.

**Antibodies**

Polyclonal rabbit anti-NECAB2 antibodies (HPA013998 and HPA014414) were generated in the human protein atlas project, HPA013998 was raised against the PrEST (aa 64-198):

VILDIFRRADKNDGKLSLEEFPQFFADGV
LNEKELEDLFTIDSDNTNHVDTKELCDYF
VDHMGQDYEDVLASLETLNHVLKAMGTYKK
VVEGGSNVQFVRFFLLKETANQIQSSLSS
VESAVEAIEEQTSQL

and HPA014414 against the PrEST (aa 239-378):

ATEDAKEEGLEAQISRLAELIGRLESKALW
FDLQQRQLSDEGTMHLQLVRQEMAVCEPQ
LSEFLDSLRQYLRGTVRNCFHITAFLVRS
DGFTVIYEFWTEEAWKRLGSPCLCKAFR
HVKVDTLSQPEALSRILVPA
Polyclonal goat anti-5HT<sub>2C</sub> antibody (ab32887) was acquired from Abcam, and monoclonal mouse anti-FLAG antibody (MAB3118) was acquired from Millipore. Secondary antibodies were HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG or rabbit anti-goat IgG.

**Gel electrophoresis and immunoblotting**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 10-12% non-stain polyacrylamide gels. Proteins were transferred to PVDF membranes using a semi-dry transfer system (BioRad) and immunoblotted using indicated antibody and then HRP-conjugated goat anti-rabbit IgG, goat anti-mouse IgG or rabbit anti-goat IgG. Immunoreactive bands were developed using ChemiDoc (BioRad).

**Immunocytochemistry**

 transiently transfected HEK293 cells were grown on fibronectin coated coverslips which were fixed in 4% paraformaldehyde and washed with PBS. Cells were permeabilized and blocked with 0.3% Triton X-100, 10% donkey serum, 5% BSA and 0.1% NaN<sub>3</sub> in 0.1 M PB for 40 min at RT and incubated with primary antibody diluted in 0.1% Triton X-100, 1% normal donkey serum and 0.1% NaN<sub>3</sub> in 0.1 M PB overnight at 4 °C. Samples were washed with PBS and incubated with secondary antibody in 0.1% Triton X-100, 0.1% NaN<sub>3</sub> and Hoechst in 0.1 M PB for 90 min at RT. Lastly, the coverslips were rinsed in water, mounted on microscope slides and examined using a V-slide microscope.

**Membrane extraction**

Cells were covered in 0.32 M sucrose dissolved in Buffer A (50 mM HEPES, 150 mM NaCl, pH 7.5), scraped from the surface and spun down at 800 rpm for 10 min. Supernatant was collected, spun down at 14,000 rpm for 30 min and the pellet was re-suspended in Buffer A. Samples were kept on ice or at 4 °C.

**Agarose beads preparation**

Antibodies were diluted in PBS, incubated with agarose beads for 1 h with rotation and cross-linked to the beads with 70 mM dimethyl pimelimidate dissolved in PBS with 0.2 M triethanolamine (wash buffer) by incubating three times for 30 min with rotation, washing in between with washing buffer. The beads were then quenched twice with 50 mM ethanolamine in PBS, washed with PBS and unbound antibodies were eluted with 1M glycine (pH 3). Beads were then washed and stored in the immunoprecipitation wash buffer at 4 °C.

**Immunoprecipitation**

For antibody optimization and CS comparison, cells were washed with cold PBS, lysed with Triton lysis buffer (1% Triton X-100, 1% n-octyl β-glucopyranoside, EDTA-free protease inhibitor, phosphatase inhibitor, in Buffer A), scraped off and centrifuged for 30 min at 12,000 rpm. Supernatant was pre-cleared with agarose beads for 1 h and incubated with antibody conjugated agarose beads (see above) overnight with rotation. Beads were washed with 0.1% Triton X-100 in Buffer A and proteins were eluted by incubating in leammli buffer for 5 min at 67 °C. All procedures were done on ice or at 4 °C unless other is stated.

For detergent optimization, cells and membranes were lysed in either CHAPS lysis buffer (1.5% CHAPS, 1% n-octyl β-glucopyranoside, 1 mM EDTA or 2 mM CaCl<sub>2</sub>, EDTA-free protease inhibitor, phosphatase inhibitor, in Buffer A) or SDC lysis buffer (0.5% sodium deoxycholate, 1% n-octyl β-glucopyranoside, EDTA-free protease inhibitor, phosphatase inhibitor, in Buffer A). When lysed with CHAPS lysis buffer, samples were washed with 0.05% CHAPS in Buffer A. When lysed with SDC lysis buffer, samples were cross-linked before lysing with 11% formaldehyde in Buffer A for 8 min at room temperature, quenched with 2.5 M glycine. Antibodies were not conjugated to agarose beads and lysate was incubated with antibodies overnight with rotation and washed with 0.1% deoxycholate in Buffer A. Otherwise everything was done as described for antibody optimization.
For MS analysis preparation, membrane fractions were prepared as described for SDC lysing buffer, washed further with 50 mM HEPES and finally washed and stored in 20 mM ammonium bicarbonate.

References


