

C. MATERIALS AND METHODS

1. Materials

1.1 *Chemicals, medium and enzymes*

Medium for cell culture such as Dulbecco's Modified Essential Medium (DMEM), N2 supplement, Fetal Calf serum (FCS), Trypsin/EDTA solution, Hanks Balanced Salt Solution (HBSS), Penicillin/ streptomycin solution were from Gibco BRL Life Technologies. Metalloprotease inhibitors GI 254023X and GW 280264X were a gift from Dr. Andreas Ludwig, Univ of Kiel, Germany. Dr. S. Roman-Roman, Aunlis pharmaceuticals, France provided RU36156. Pharmacological agents NMDA, Kainate, DNQX, APV, PMA, nimodipine and ω -conotoxin MVIIC were from Tocris. From Sigma, we procured 1, 10 phenanthroline, MG132, cycloheximide, actinomycin D. TAPI-1 and TAPI-2 were purchased from Peptides International. TFP, U0126, Ilomastat, FK-506 were from Calbiochem. NMDA, Kainate, ω -conotoxin MVIIC, APV and RU 36156 were dissolved in distilled water. Dimethylsulfoxide was used as a solvent for dissolving nimodipine, U0126, TAPI-1, 1, 10 phenanthroline, Ilomastat, DNQX, FK-506, TFP, MG132, actinomycin D, GI 254023X and GW 280264X with the final concentration of 0.1% DMSO in cell cultures. TAPI-2 and cycloheximide were dissolved in ethanol with a final concentration of 0.1% ethanol in cultures. All the standard laboratory chemicals, acids and bases were from Merck, Roth, Serva, Fluka or Sigma.

The restriction enzymes used in molecular biological studies (for sub cloning) were from Boehringer Mannheim, Gibco BRL Life Technologies, MBI Fermentas, Pharmacia Biotech, New England Biolabs and USBTM Amersham LIFE SCIENCE.

1.2 *Consumables and special appliances*

Eppendorf tubes were from Eppendorf, AG (Germany). Cell culture plates, flasks and cell scrapers were from Techno Plastic Products, AG, (Switzerland). All the glass wares were from Scott, AG (Germany). Syringes and needles were procured from B.Braun Melsungen, AG (Germany). Filters used for sterile filtration were from Millipore (USA). The tools for dissection such as scissors and tweezers were obtained from A.Dupoint & Fils (Germany).

Chemiluminescent films were from Roche (Switzerland). Solutions for chemiluminescence blot development i.e., Fixative and Developer solutions were from AGFA (Belgium). Cell counter was from Coulter Electronics, United Kingdom (Model: Coulter Z1). Ultracentrifuges and their respective rotors were from Dupont Company, USA (Sorval Models: RC 5B, Ultra Pro 80 and RC 120EX). The chemiluminescent blot developing machine was from AGFA, Belgium (Model: CURIX 60). Spectrophotometer was of Eppendorf AG, Germany (Model: Biophotometer). Microscopes used were from Zeiss, Germany (Model: Axiovert 135; Telaval 31).

1.3 Softwares used

Microsoft Excel, version 2002 was used for statistical calculations such as statistical significance values, mean, standard error and standard deviation. Graphs were made by using Micrococal Origin, version 5. Chemiluminescent gel bands were refined using Adobe Photoshop, version 7. Quantification of the bands of Western blots was done by Image J, NIH Image.

1.4 Antibodies

Primary antibodies

Monoclonal anti- PSD 95	-Clone K28/86.2; Upstate biotechnology
Monoclonal anti- γ -tubulin	-Clone GTU-88; Sigma
Monoclonal anti-HA	-Clone 3F10; Roche
Monoclonal anti-Flag	-M2 and M5; Sigma
Polyclonal anti-GluR 2/3	-Chemicon, a gift from Dr. L.R. G. Britto; used at 1:500
Monoclonal F11 (Contactin1)	- 1 μ g/ml (No.5), obtained from Prof. F. G. Rathjen (Rathjen et al., 1987)

Commercially procured primary antibodies were diluted and used according to the manufacturer instructions.

CALEB primary antibodies

Monoclonal 4/1 (mAb 4/1)	Working dilution- 0.065 μ g/ml
Monoclonal 1-2B/8 (mAb 1-2B-8)	Working dilution- 0.1 μ g/ml
Monoclonal 3-2G/10 (mAb 3-2G-10)	Working dilution- 0.1 μ g/ml

Rabbit polyclonal 462 (pAb 462)	Working dilution- 1 µg/ml
Rabbit polyclonal C5 (pAb C5)	Working dilution- 3 µg/ml

Antibodies against chick CALEB (mAb 4/1, pAb C5) and F11 were obtained from Prof. Rathjen. Antibodies against the cytoplasmic domain of CALEB (mAb 8-1B-8; mAb 3-2G-10) were provided by Dr. Aleksei Babich. Dr. Margret Moré provided the pAb 462.

Sl.no	Antibody	Species reactivity	Epitope recognised
1	mAb 4/1	Chick	280-285 aa
2	pAb C5	Chick	Nter-280 aa
3	pAb 462	Mouse	48-420 aa
4	mAb 3-2G-10	Chick & Mouse	532-543 aa
5	mAb 8-1B-8	Chick & Mouse	537-544 aa

Table 1:Antibodies list. The table provides the list of antibodies used in the study and their species specific epitopes. “aa” denotes the amino acids. The first four antibodies were generated against complete CALEB and the last two were generated against peptides in the cytoplasmic domain.

Secondary antibodies

Goat anti-mouse-Horse Radish Peroxidase (HRP)	Working dilution- 1:25,000
Goat anti-rabbit-HRP	Working dilution- 1:40,000
Goat anti-mouse/rabbit-Alkaline Phosphatase (AP)	Working dilution- 1:4000
Goat anti-mouse-Cy3	Working dilution- 1:500

The secondary antibodies were obtained from Dianova.

“Protease inhibitor cocktail” was composed of PMSF (200 µM), Pepstatin (10 µM), Aprotinin (0.5 µM) and Leupeptin (10 µM) (final concentration).

1.5 cDNAs

Plasmids encoding chick CALEB (aa275- aa595; Clone 12; pcDNA6-preprotrypsin/Flag/ch.CALEB/Myc/His) were obtained from Prof. F. G. Rathjen and Mrs. Mechthild Henning. Plasmids encoding for ADAM 10-Flag (active) and ADAM 10-HA (dominant negative) were from Prof. Paul Saftig (Univ. of Kiel, Germany).

1.6 Competent cells (Bacteria)

For the purpose of sub cloning and transformation DH5 α strain of *E.coli* were used (obtained from Prof. F. G. Rathjen). These bacterial strains were made chemo-competent for transformations.

1.7 Animals, cells (primary and cell lines), tissues

Chicken embryos from fertilized eggs (VALO SPF eggs, Lohmann Tierzucht GmbH, Germany) of White leghorn species (*Gallus domesticus*) were used for preparing retinae and other tissues, for cultures and developmental expression respectively. Pigmented mouse strain C57Bl/J6 (The Jackson laboratory) was used for analysing CALEB developmental expression profile. CALEB knock-out mouse had been backcrossed for seven generations with C57Bl/J6 mice. The CALEB knock out mice were provided by Dr. Margaret Moré.

The fibroblast cell line COS7 was used.

2. Methods

2.1 Cell culture methods

Established cell lines were maintained and primary cell cultures prepared as follows:

2.1.1 Maintenance of cell line

Cell lines of different origins were thawed from liquid nitrogen and plated in 10 ml of Cell culture medium. Cells were allowed to grow until 90% confluence. Medium of the confluent cultures was removed, followed by washing with PBS. Cells were incubated with trypsin/EDTA solution for 1-2 min then flushed from the bottom of the culture flask with Cell culture medium. Cells were finally plated at a density of $1-5 \times 10^5$ cells/ml in 24 well dishes and grown in cell culture medium. Transfection of the cells was done at 75-80% confluence.

Cell lines of various origins after usage were washed with PBS, trypsinized with trypsin/EDTA and resuspended in Freezing medium. These cells were then frozen in aliquots of 1 ml in liquid nitrogen.

Solutions:

PBS: 137 mM NaCl
2.7 mM KCl
1.5 mM KH₂PO₄
6.5 mM Na₂HPO₄

Cell culture medium: DMEM containing 10% FCS
Penicillin/ streptomycin (final con. 100 units/ml)

Freezing medium: DMEM
20% FCS
10% DMSO

2.1.2 Primary cell culture

Fertilized chicken eggs were incubated at 37⁰C humidified (65% humidity) egg incubator and retinae were dissected at Hamilton & Hamburger Stage 34 (Hamburger and Hamilton, 1951) corresponding to E8 (embryonic day 8). Dissected retinas were collected in cold DMEM and incubated in Trypsin (Boehringer Mannheim) for 20 min at 37⁰C in a water bath. Trypsinized tissue was mechanically triturated with fire polished Pasteur pipette in DMEM, followed by centrifugation. This step was repeated, to ensure the presence of dissociated cells. Finally the single cell suspension was diluted in chick retina culture medium and counted either in Coulter cell counter or Neubauer chamber slides. Cells were plated on precoated poly-L-lysine (Sigma) dishes at a density in 1.5x10⁶ cells/ml per well of a 24-well-plate in DMEM/N2 (Gremo et al., 1984) and cultivated for 3 days in vitro (DIV) at 37⁰C.

Solutions:

Chick retina culture medium: DMEM with N2 supplement

Trypsin: 1 mg Trypsin/ ml HBSS
Sterile filtered

Poly-L-lysine: 100 µg Poly-L-lysine/ ml distilled water
Sterile filtered

2.2 Biochemical methods

2.2.1 Electrophoresis and Western blot

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cell or tissue lysates were heat denatured with Laemmli buffer (2X). The denatured samples were resolved on polyacrylamide gels containing 7.5-15% of acrylamide. The samples were electrophoresed at 110-140V for 1-1½ hr at room temperature with electrophoresis buffer in miniapparatus, Mini Protean II from BioRad. The separated proteins were electroblotted from the gel by electrical transfer onto the nitrocellulose membrane (Schleicher & Schuell) (equilibrated in transfer buffer) at 326 mA for 1-2 hr in cold transfer buffer. The 15% acrylamide gels were electro transferred for 2 hr and the 7.5% acrylamide gels were electro transferred for 1 hr. The electroblotted nitrocellulose membrane was blocked by incubating with milk powder solution or bovine serum albumin (blocking solution) for 1 hr at room temperature. The membrane was then incubated with primary antibody appropriately diluted in blocking solution for 2 hr at room temperature, or overnight at 4°C. After incubation, the membrane was washed three times with PBST 10 min each at room temperature, followed by incubation with diluted secondary antibody for 1 hr at room temperature. Secondary antibodies tagged with alkaline phosphatase (AP) were diluted at 1:4000 and those tagged with horseradish peroxidase (HRP) were diluted at 1:25,000-40,000 in blocking solution. The membrane was washed for 15 min each in three changes of PBST with a final wash for 30 min (Laemmli, 1970; Towbin et al., 1979). Membranes incubated with the AP conjugate were developed with developing solution. The colour development reaction was stopped by using stop buffer to prevent excess development. Membranes incubated with HRP tagged antibody were developed using “Super signal West Dura Extended Duration” substrate (Pierce) according to the manufacture’s instruction. The membrane was developed by chemiluminiscent technique using “Lumi-film Chemiluminiscent” detection film.

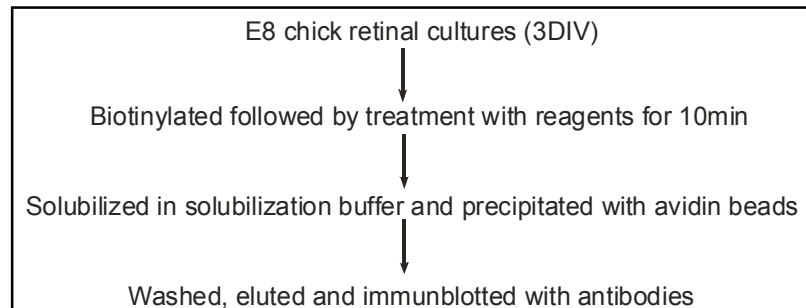
Solutions:

5X Laemmli buffer (stock):	60 mM Tris/Cl, pH 6.8
	10% SDS
	10% β-mercaptoethanol
	50% Glycerol

	1.5% Bromophenol blue
Electrophoresis buffer:	25 mM Tris/Cl, pH 8.3 190 mM Glycine 0.1% SDS
Transfer buffer:	25 mM Tris/Cl, pH 8.3 190 mM Glycine 20% Methanol
Milk powder solution:	2.5% skimmed milk powder 0.5% Tween 20 Solution made in PBS
BSA blocking solution:	4% BSA 0.5% Tween 20 Solution made in PBS
PBST:	Phosphate buffer saline 0.5% Tween 20
Developing solution:	16.5 µl BCIP solution (stock) 33 µl NBT solution (stock) 10 ml of AP buffer
AP buffer:	100 mM Tris/Cl, pH 9.7 5 mM MgCl ₂ 0.01 mM ZnCl ₂ Solution made in distilled water
BCIP solution (stock):	5-Bromo-4-chloro-Indolyl-phosphate (Biomol) 50 mg/ml in DMF
NBT solution (stock):	Nitro-blue-tetrazoliumchloride (Biomol) 50 mg/ml in 50% DMF
Stop buffer:	20 mM Tris/Cl, pH 8.0 5 mM EDTA Solution made in distilled water

Agarose gel electrophoresis

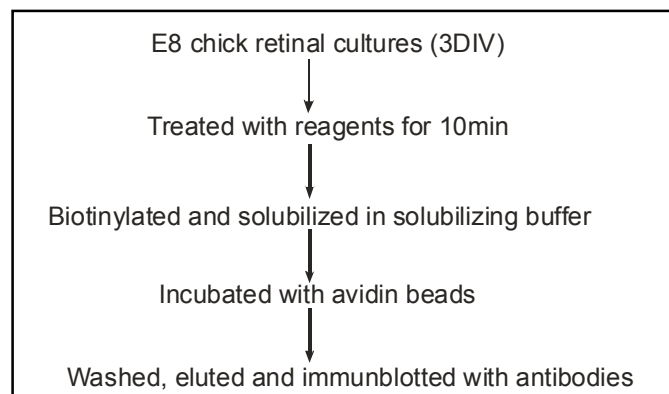
1989;Meyer-Franke et al., 1998). The retinal cells were initially washed twice with cold HBSS and then incubated with cold biotinylation solution for 40 min at 4°C. Cells were washed once with ice cold HBSS, followed by quenching free biotin with DMEM for 10 min (Coligan et al., 1994).



Scheme 1:**Pre-biotinylation protocol.** Schematic representation of the pre-biotinylation experiment protocol, for cell surface biotinylation of chick retina cultures before incubation with various pharmacological agents.

The cells were then incubated in DMEM/N2 for 10 min. Biotinylated chick retinal cells were either left untreated or treated with pharmacological agents and then lysed with solubilizing buffer for 10 min. Biotinylated surface proteins were captured with 12-14 μ l streptavidin beads (Pierce) by immunoprecipitation and eluted by boiling the streptavidin beads in 50 μ l of Laemmli buffer. The proteins were separated by SDS-PAGE and Western blotted with antibodies (Scheme 1).

In post-labelling experiments, the retinal cells were biotinylated as described above, but only after treatment with various reagents. After biotinylation the cells were lysed immediately in solubilizing buffer; precipitated with streptavidin beads, eluted by boiling the streptavidin beads in Laemmli buffer and the proteins resolved in SDS-PAGE immunoblots (Scheme 2).

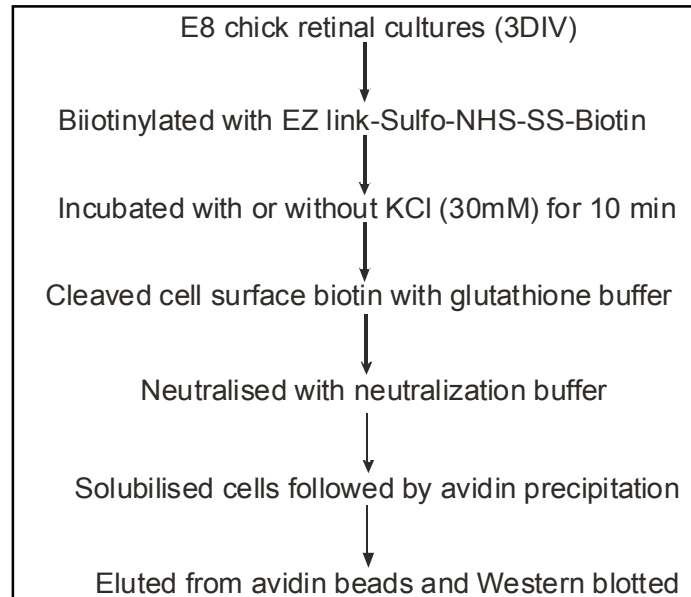


Scheme 2:**Post-biotinylation protocol.** Schematic representation of the post-biotinylation experiment protocol, for cell surface biotinylation of chick retina cultures after incubation with various pharmacological agents.

Solution:

Biotinylation solution: 5 mg EZ-link Sulfo-NHS-LC-Biotin
dissolved in 250 μ l of DMSO
finally added to 10 ml of HBSS

2.2.6 Biotin Internalization Assay



Scheme 3: **Biotin internalization protocol.** Schematic representation of the cell surface biotin internalization protocol, which was done in chick retina cultures to rule out an enhanced CALEB internalization upon KCl treatment.

For the analysis of surface internalization of proteins, chick retinal cell cultures (3DIV) were pre-treated with 100 μ g/ml of leupeptin (Fluka) in DMEM/N2 for 30 min to prevent degradation of internalized proteins. The cells were then washed with ice cold HBSS followed by biotinylation with EZ-link Sulfo-NHS-SS-Biotin (300 μ g/ml, Pierce) at 37⁰C for 2 min (Ehlers, 2000). After washing with HBSS, unbound biotin was quenched with DMEM, followed by incubation in DMEM/N2 at 37⁰C for 10 min further. The medium was then replaced by fresh medium with or without KCl (30 mM) for 10 min. Cellular trafficking was halted by rapid cooling at 4⁰C and the cells were either solubilized or the biotin on the cell surface was stripped off by incubating cells twice with Cleavage buffer at 4⁰C for 15 min each. The glutathione containing cleavage buffer was subsequently neutralized with neutralization buffer; cells lysed; biotinylated proteins captured with streptavidin beads as described above, eluted and immunoblotted in reducing conditions (Scheme 3).

Buffers:

Cleavage buffer: 150 mM Glutathione in 150 mM NaCl,
pH 8.75

Neutralization buffer: 50 mM iodoacetamide in PBS

2.2.7 Identification of a released CALEB fragment

Conditioned supernatants of chick retina cultures (3DIV) which were either treated with 30 mM KCl or left untreated for 10 min were collected. The conditioned supernatants were centrifuged at 10,000 g for 20 min and 100,000 g for 30 min, after addition of protease inhibitor cocktail. To the supernatants collected after centrifugation steps, equal volume of 10% Tri-chloro acetic acid solution was added, mixed, incubated on ice for 30 min. Then the solution was centrifuged for 1 hr at 100,000 g and the pellet (precipitated proteins) washed three times with 70% ethanol (Tsakadze et al., 2004). Finally, the pellet was dissolved in Laemmli buffer and resolved by Western blot.

2.2.8 Characterization of membrane associated protease that cleaves CALEB

The supernatant was removed from chick retina cultures (3DIV). The cells washed and incubated with 5 volumes of a hypotonic solution for 15 min on ice. The cells then were scraped and homogenized using Dounce homogenizers and needle & syringes. This was followed by an initial centrifugation at 2000 g for 5 min, the supernatant collected and centrifuged further at 10,000 g for 1 hr at 4^oC (Ito et al., 1999b). The pellet containing the crude membrane fractions was dissolved in SDS-PAGE sample buffer, boiled followed by electrophoresis and Western blot or followed as mentioned in section 2.2.9 (below).

Solution:

Hypotonic solution: 20 mM HEPES pH 7.5
10 mM KOAc
1.5 mM MgOAc

2.2.9 *In-vitro* CALEB cleavage reaction

Membrane fractions were prepared as mentioned above and incubated with 10 volumes of ice cold 1 M NaCl in 30 mM Tris/Cl, pH 7.2 on ice for 15 min. The membranes were then

collected by centrifugation at 10,000 g for 15 min at 4°C. The pelleted membranes were reconstituted in ice cold 30 mM Tris/Cl pH 7.2. These membrane fractions were incubated with or without TAPI-1 (broad spectrum metalloprotease inhibitor) for 2 hr at 37°C. The reaction of the metalloprotease was stopped by boiling the samples with Laemmli buffer. Western blots were made from these probes using CALEB cytoplasmic antibody (mAb 1-2B-8) (Ito et al., 1999b).

2.3 Molecular biological methods

2.3.1 Preparation of competent cells

Chemo-competent DH5- α bacterial cells were prepared as mentioned below. A single colony of DH5- α strain was incubated in 3 ml of Luria-Bertani (LB) broth containing potassium chloride (10 mM) and magnesium sulphate (20 mM). One ml of this culture was added to 100 ml of LB liquid medium with potassium and magnesium salt and incubated at 37°C to make a broth culture. The cells were grown until an OD 0.4-0.5 was reached at 660 nm. The cells were incubated for 10 min on ice and centrifuged at 3000 rpm for 10 min at 0°C. The pellet was resuspended in 30 ml of TFB I solution. The suspension was incubated for 10 min on ice followed by further centrifugation for 10 min at 0°C. Finally the pellet was resuspended in TFB II buffer and incubated for 10 min and the competent bacteria were stored at -70°C. The DH5- α competent cells were stored for a day at -70°C before using the cells for transformation.

Solutions:

LB broth:	10 g/L tryptone 10 g/L NaCl 10 g/L yeast extract Solution made in distilled water, autoclaved
TFB I:	30 mM potassium acetate 50 mM manganese chloride 100 mM RbCl ₂ 10 mM CaCl ₂ 15% Glycerin pH adjusted to 5.8 with 0.2 N HOAc Solution is sterile filtered

TFB II:	10 mM Na-MOPS
	75 mM CaCl ₂
	10 mM RbCl
	15% Glycerin
	pH adjusted to 7 with NaOH
	Solution is sterile filtered

2.3.2 Transformation

Bacterial transformation was done to amplify the cDNA. Competent bacteria were thawed on ice. Approximately, 0.1 µg plasmid DNA was incubated with the bacteria for 20 min on ice. Bacterial cells along with the DNA were incubated at 42⁰C for 2 min (heat shock step) and then placed on ice for 1-2 min. To help the bacterial cells recover from heat shock, the cells were briefly incubated in 400 µl of LB broth (with KCl and MgCl₂) and incubated at 37⁰C for 1 hr on a shaker. At the end of 1 hr incubation 100 µl of LB broth with the bacterial cells (from the 400 µl of LB broth) was added to the LB plate with ampicillin (100 mg/ml) (to select bacteria containing the plasmid conferring ampicillin resistance). Plates were incubated at 37⁰C overnight, and individual colonies were picked and tested for the presence of the plasmid by the following protocol: Briefly, single colonies were picked and incubated in 3 ml of selection medium (LB broth and ampicillin antibiotic selection) for 8 hr at 37⁰C on shaker. Plasmid DNA was isolated from the 3 ml cultures by kits of Qiagen and restriction enzyme digestion reaction was done to confirm the presence of the insert. Larger cultures were grown by adding 100 µl of 3 ml positive cultures (containing the plasmid of interest) to 100 ml of LB broth with the antibiotic selection (ampicillin) and incubated on a shaker overnight at 37⁰C (Hanahan, 1985).

2.3.3 Plasmid DNA preparation

DNA isolation was done from 3 ml and 100 ml bacterial cultures by using the Qiagen plasmid kit, following the provided instruction manual. The DNA amount obtained was quantified by spectrophotometer at 260 nm.

2.3.4 Restriction enzyme digestion

The plasmid DNA was digested using restriction enzymes that have been used to insert the fragment in the plasmid (to check the presence of the insert of interest). The DNA of

interest was incubated with appropriate restriction endonuclease according to the parameters suggested by the manufacturer, briefly

1 µg of DNA of interest

1 µl of each restriction enzyme of interest

2 µl of 10x restriction buffer

The final volume is made to 20 µl total reaction volume with autoclaved water. The reaction is incubated for 2 hr at 37°C. The samples are then loaded in agarose gel and the molecular weights compared with DNA standard marker (Ausubel et al., 1994a).

2.3.5 Transfection of cDNAs to cell lines

The plasmid DNA isolated was used for transfections of cell lines. Cells were transfected using Lipofectamine 2000 (Invitrogen) following the guidelines mentioned in the instruction manual. After transfections the cells were incubated with medium containing DMEM/10% FCS with antibiotics for 24 hr before checking for the expression levels.

2.4 *Histological methods*

2.4.1 Tissue sectioning

Embryonic chick brain and retinae were collected from E11 fertilised eggs in PBS. The tissues were fixed with 3.7% formaldehyde in PBS containing 8% sucrose solution for 8 hr at 4°C on a slow shaker. Tissues were then incubated in 30% sucrose solution in PBS (for cryoprotection) at 4°C for over night. Tissues were embedded in Tissue tek (Sakura). Acid washed (10% HCl) glass slides were coated with gelatine and dried at 37°C (von Bohlen et al., 1999).

Cryosections of 16 µm and 18 µm were made from the whole embryonic chick brain and retinae respectively. The sections were collected on gelatine pre-coated glass slides, stored in -20°C and stained the following day.

Solution:

Coating solution: 5 g gelatine

Solutions:

Blocking solution: 10% Goat serum in PBS
0.1% Tween 20

Antibody dilution solution: 5% Goat serum in PBS
0.1% Tween 20

Wash buffer: PBS with 0.1% Tween 20

Fixative: 3.7% formaldehyde solution in PBS

2.5 Immunocytochemistry

2.5.1 Fixation and immunostaining

Chick retina cultures were made on poly-L-lysine precoated cover slips. After 3 DIV, the medium was removed, the coverslips washed with PBS and incubated with primary antibody (diluted to 3-5 $\mu\text{g/ml}$ in wash buffer) for 1 hr at room temperature. The coverslips were then washed three times with washing buffer followed by fixation with ice cold fixative for 10 min at room temperature. After washing twice with wash buffer the cells were incubated with secondary antibody for 30 min at room temperature. The secondary antibody tagged with Cy3 was diluted 1:500 in wash buffer. The coverslips were washed twice and the cells were fixed for 10 min in ice cold fixative. The cells were washed in wash buffer; mounted on glass slides with Mowiol and observed under microscope.

Solutions:

Fixative: 3.7% formaldehyde in ice cold PBS

Wash buffer: PBS containing 0.1% BSA solution

Poly-L-lysine: 100 μg Poly-L-lysine
1 ml of distilled water
Sterile filtered

2.6 Post-synaptic density (PSD) preparation

2.6.1 Animals and reagents

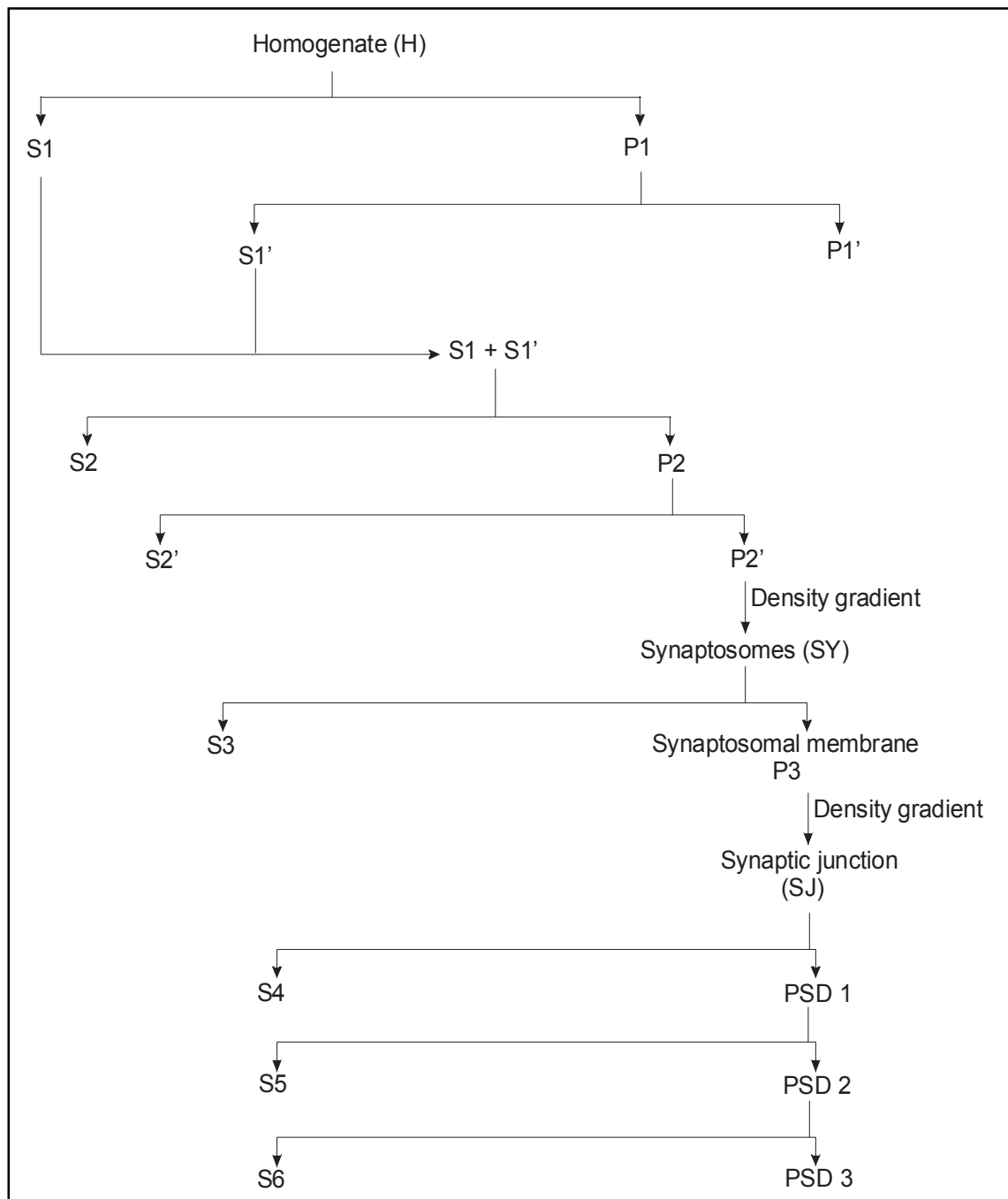
C57Bl/6J adult mice were used for making the PSD fractions. A total of 30 mice were decapitated and brains collected in ice cold PBS with protease inhibitors. Approximately, 4-5 g brain was used as the starting material.

2.6.2 Protocol for PSD preparation

Brains were homogenized in Buffer A (Homogenization buffer) containing protease inhibitors with 12 strokes of motor operated Teflon-glass homogeniser. The final volume of Buffer A used was 50 ml for 5 g of brain weight. The homogenate was centrifuged for 10 min at 1000 g in Sorval RC 5 centrifuge with SS-34 rotor, yielding S1 and P1.

The initial steps of homogenization were repeated with the pellet P1, i.e., stroking with homogenizer and centrifugation giving rise to S1' and P1'. The supernatant S1 and S1' were combined and centrifuged at 12000 g for 15 min. From this centrifugation we obtained S2 and P2 (crude membrane fraction). The pellet P2 was homogenized with 6 strokes in the presence of Buffer A with protease inhibitor. Homogenized P2 was centrifuged at 12000 rpm for 20 min. The pellet P2' was resuspended in 6-7 ml of Buffer B. This whole mixture of Buffer-B and P2' pellet was placed on top of the sucrose gradient of 0.85 M, 1 M and 1.2 M sucrose. The gradient was centrifuged at 85,000 g/ 2 hr. The band of interest separates in the gradient between 1.2 M and 1 M sucrose and is rich in synaptosomes. The synaptosomes were stirred slowly in the presence of 5 volumes of Tris/Cl (with 1 mM DTT) for 30 min at 0°C followed by centrifugation at 33,000 g/ 30 min in Sorval RC5, resulting in very rich synaptosomal membrane fraction (P3) and the supernatant (S3). P3 was dounced with homogenizer manually in the presence of 6-7 ml of 5 mM Tris/HCl. The lysed synaptosomal membrane fraction was placed on a "Step gradient" of 1 M, 1.5 M and 2 M sucrose and centrifuged for 2 hr at 85,000 in Sorval AH-629. The synaptic junctions were isolated from the interface of sucrose gradient 1.5 M and 2.0 M. To the synaptic junctions added 30 ml buffer B with 30 ml (60 ml/10 g of starting material) of buffer C along with 2 mM DTT and incubated for 15 min with slow stirring on ice. This mixture of solutions along with the synaptic junctions was centrifuged at 33,000 g for 30 min in Sorval SS 34 to obtain the first PSD fraction. PSD-I (P4) with 30 ml of Buffer B and 30 ml of Buffer C was stirred on ice for 15 min. This was centrifuged at 20,500 rpm

for 30 min in order to enrich PSD-I, and then to obtain PSD-II (P5). The PSD-II is further resuspended in 1 ml of Buffer B, overlaid on a sucrose cushion of 1.5 M. This was centrifuged at 200,000 g for overnight in Sorval AH-629. . The final PSD, that is PSD-III (P6) was obtained and resuspended manually in 2 ml of 50 mM HEPES pH 7.2 (Carlin et al., 1980) (Scheme 4). Protein estimation was done of all the fractions obtained and equal amount of protein were resolved in immunoblots.



Scheme 4: **Post-synaptic density preparation.** Mouse adult brain were collected and the protocol mentioned above was followed to get the synaptosomes, synaptic junctions as well as to get purified post-synaptic density fractions.

Solutions:

Protease Inhibitors:	200 μ M PMSF 10 μ M Pepstatin 0.5 μ M Aprotinin 10 μ M Leupeptin
Sucrose gradient:	0.85 M Sucrose/ 5 mM Tris/ HCl pH 8.1 1.0 M Sucrose/ 5 mM Tris/ HCl pH 8.1 1.2 M Sucrose/ 5 mM Tris/ HCl pH 8.1 1.5 M Sucrose/ 5 mM Tris/ HCl pH 8.1 2.0 M Sucrose/ 5 mM Tris/ HCl pH 8.1
Buffer A:	0.32 M sucrose 5 mM HEPES, pH 7.4
Buffer B:	0.32 M sucrose 5 mM Tris/ HCl, pH 8.1
Buffer C:	0.32 M sucrose 12 mM Tris/ HCl, pH 8.1 1% Triton

2.7 Superior colliculus incubation experiments

Superior colliculus of P10, C57Bl/6J mice were dissected in ice cold artificial cerebrospinal fluid (ACSF). The tissues were then incubated at room temperature in 50 ml of ACSF for 2 hr with continuous bubbling with CO₂/O₂ (95%/ 5%) containing 10 mM KCl or 50 μ M APV + 10 μ M DNQX or 20 μ M bicucullin or left untreated. The superior colliculus was then homogenized in solubilization buffer and protein content estimated. Equal amounts of proteins were resolved and analysed by Western blot with pAb 462 and mAb γ -tubulin.

Solution:

ACSF:	125 mM NaCl
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4 mM KCl

10 mM glucose

1.25 mM NaH₂PO₄

25 mM NaHCO₃

0.5 mM CaCl₂

2.5 mM MgCl₂

solution was made in distilled water