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It was observed in this laboratory that a glycoprotein of molecular weight nearly 200kDa decreased proportionately with the increase in duration of REM sleep deprivation (Mishra 2004, unpublished data). This decrease in the level of the protein reverted back to its normal serum level after recovery from REM sleep deprivation. These observations strongly suggest that the protein can possibly serve as a marker for REM sleep deprivation. However, for this purpose the biochemical identity of the protein was required to be known.

To achieve the objectives of the present study, the 200kDa protein was purified in its native form using the chromatographic techniques. Thereafter, antibodies were raised against the purified protein. The antiserum was used to detect the protein immunohistochemically in the various brain regions. The physiological modulation of the protein by norepinephrine (NE) was studied by injecting adrenergic receptor agonists and antagonists (intraperitoneally) and the effects on changes in the level of this protein were observed. Further, transcriptional regulation of the protein was also checked. Various procedures and protocols followed in this study have been discussed in the following sections.

ANIMALS USED

Male Wistar rats weighing 220-250gm were used in the study. The animals were procured from the central animal house facility of Jawaharlal Nehru University (JNU). The project had the approval of the University Animals Ethics Committee, JNU. The animals were kept in semi-transparent polypropylene cages and maintained under 12:12 dark-light cycle in a temperature controlled room. Food and water were provided *ad libitum*.

REM SLEEP DEPRIVATION

The flower pot method was used to deprive the rats of REM sleep. This method is also known as water tank or platform or pedestal technique. It was designed by Jouvet et al. for depriving cats of REM sleep (Jouvet et al. 1964) and has since been used extensively for cats, rats and mice (Hicks et al. 1977; Oniani et al. 1988; Gulyani et al. 2000). In this method, animals are maintained on a small raised platform, typically inverted flowerpot, surrounded by water (Fig VII).
The animals can sit, crouch and move around freely on this island, however, they cannot have enough room for relaxation. As soon as the animals experience REM sleep, due to atonia in the postural muscles, they cannot stay on the small platform and fall or tend to fall into the surrounding water, preventing them to go into REM sleep. Thus, in this method the animals can have NREM sleep but not REM sleep. This technique is very effective, inexpensive as well as procedurally simple and at the same time allows selective REM sleep deprivation to a large number of animals simultaneously. A relatively large platform enables the animal to curl up and have both NREM and REM sleep under similar surroundings and is most effectively used as a control.

The platform technique is most widely used for REM sleep deprivation studies. The optimum ratio of surface area of the small platform to animals' body weight for maximal REM sleep deprivation has been suggested not to be more than $14 \text{cm}^2/100 \text{gm}$. For large platform control, the suggested ratio is at least $58 \text{cm}^2/100 \text{gm}$ (Mendelson 1974). Mendelson compared sleep patterns for 96 hrs from four groups of normal and control rats (Mendelson 1974). It was concluded that the method was quite effective for longer duration deprivation studies, although, not very effective for studying REM sleep deprivation for up to 24 h.

Flower Pot method for REM sleep deprivation

![Flower Pot method for REM sleep deprivation](image)

**Fig VII**
Materials and Methods

Each set of experiment consisted of one rat each of free moving control (FMC), REM sleep deprived (REMSD), large platform control (LPC) and recovery (REC) group. The FMC rats were housed in their dry home cages. The REM sleep deprived rats were deprived of REM sleep for 6 days by the platform method. To rule out the non-specific effects, some rats were maintained on larger (13cm diameter) platforms and these served as large platform controls (LPC). After REM sleep deprivation a few animals were kept back in their home cages for 3 days in order to recover from the effects of REM sleep deprivation. These served as recovery (REC) rats. All the experiments were done in three sets, each containing four rats (one rat each as FMC, REMSD, LPC and REC).

PROTEIN PURIFICATION

Serum isolation:

Serum was prepared according to the method described in the antibody manual (Harlow and Lane 1988). Briefly, blood samples were collected from the tail vein of the rat as per the requirement. The blood was allowed to clot for 2 hours at room temperature. The clot was then removed by detaching it from the sides of the collection vessel. The vial was left overnight at 4°C to allow the clot to shrink. The fluid part was then removed from the clot and centrifuged at 10,000 rpm for 10 min at 4°C in a cooling centrifuge (Biofuge, Fresco). After preparation, the serum was aliquoted and stored at -70°C till further use.

Principle of chromatography:

One of the most frequently employed methods for separation and purification of biological compounds is chromatography. The basis of all forms of chromatography is the partition or distribution coefficient (Kd), which describes the way in which a compound distributes itself between two immiscible phases. For a compound distributing itself between equal volumes of two immiscible solvents A and B, the value for distribution coefficient is constant at a given temperature and is given by the expression:

\[
\frac{\text{Concentration of solvent A}}{\text{Concentration of solvent B}} = K_d
\]
The distribution of a compound can be described not only in terms of its distribution between two solvents, but also by its distribution between any two phases, such as solid/liquid or gas/liquid phases. The term effective distribution coefficient is defined as the total amount (distinct from the concentration) of substance present in one phase divided by the total amount present in the other phase. Principally, all chromatographic methods consist of two phases. One is the stationary phase which may be solid, gel, liquid or a solid/liquid mixture which is immobilized. The second is the mobile phase which may be liquid or gaseous and flows over or through the stationary phase. The choice of stationary or mobile phases is made such that the compounds to be separated have different distribution coefficients. This may be achieved by setting up:

(i) an adsorption equilibrium between a stationary solid and a mobile liquid phase (adsorption chromatography)

(ii) a partition equilibrium between a stationary liquid (or semi-liquid) and a mobile liquid phase (countercurrent chromatography and partition chromatography)

(iii) a partition equilibrium between a stationary liquid and a mobile gaseous phase (gas-liquid chromatography)

(iv) an ion-exchange equilibrium between an ion exchange resin stationary phase and a mobile electrolyte phase (ion-exchange chromatography)

(v) an equilibrium between a liquid phase inside and outside a porous structure or molecular sieve (exclusion chromatography)

(vi) an equilibrium between a macromolecule and a small molecule for which it has a high biological specificity and hence affinity (affinity chromatography)

In the present study, purification of the protein of interest having mol wt of approx 200kDa was achieved by using a combination of different chromatographic methods.
Sample preparation:

Proper adjustment of the sample pH and ionic strength is critical for consistent and reproducible results when using chromatographic techniques. The sample must be exchanged into the appropriate application buffer. This can be achieved by exchanging it in the application buffer using various desalting columns or desalting gels. Alternatively, the sample can be dialyzed against the application buffer. Dialysis is a well-established separation method that allows for buffer exchange and low molecular weight contaminant removal from sample solutions without significant loss of the macromolecule of interest. It is based on the diffusion of small molecules in a sample through a semi-permeable membrane into a second liquid or dialysate. Diffusion is a process that results from thermal, random movement of molecules from an area of higher to lower concentration. The rate of diffusion of a molecule is directly proportional to its concentration and inversely proportional to its mol wt. The higher the concentration, the greater the probability that the molecules will come in contact with the membrane and diffuse across it to the other side. The larger the molecule, the slower its movement in solution and lesser the chance that it will collide with and diffuse through the membrane even if it is small enough to pass through the pores. Dialysis rate is directly proportional to the surface area of the membrane and inversely proportional to its thickness. A typical dialysis procedure followed for protein separation (PROTOCOL 1) in the present study was as follows:

1. The sample was dialyzed against the buffer of interest for 1-2 hours at room temperature.
2. The dialysis buffer was changed and dialysis continued for another 1-2 hours.
3. The dialysis buffer was changed again and dialyzed overnight at 4°C.

The volume of the dialysis buffer was at least 200-fold than the sample volume to enhance the differential gradient. Subsequently, the dialyzed sample (normal rat serum sample in present study) was applied on the chromatographic column.
I. **Cibacron Affi-Gel Blue Chromatography:** Affi-Gel blue gel has been used to separate and purify a number of different serum and plasma proteins. The purification using this column is based on the principle of affinity chromatography. Purification by affinity chromatography is unlike all other forms of chromatography in that it does not rely on the differences in the physical properties of the molecules to be separated. Instead, it exploits the unique property of extremely specific biological interactions to achieve separation and purification. As a consequence, affinity chromatography is theoretically capable of giving absolute purification, even from complex mixtures, in a single process. The technique was originally developed for the purification of enzymes, but it has been extended to nucleotides, nucleic acids, immunoglobulins, membrane receptors and even whole cells and cell fragments.

Affi-Gel blue affinity gel is a beaded, cross-linked agarose gel with covalently attached Cibacron Blue F3GA dye (a reactive anthraquinone-type dye). It contains \( \geq 1.9\) mg dye per ml of gel, and has a capacity for albumin binding of greater than 11mg/ml. Affi-Gel blue gel purifies a large range of proteins from widely divergent origins. The blue dye functions as an ionic, hydrophobic, aromatic or sterically active binding site in various applications. Proteins that interact with Affi-Gel blue gel can be bound or released with a high degree of specificity by manipulating the composition of the eluent buffers.

Affi-Gel blue gel provides a simple first step in the purification of many serum proteins by removing albumin, the major serum constituent. The binding of albumin is so strong that a high concentration of salt or chaotropic reagent is required to desorb the albumin. Other serum proteins either do not bind to Affi-Gel blue gel or can be eluted with relatively low concentrations of salt.

**Buffer Formulations for Albumin removal procedure:**

* **A Buffer:** 20mM phosphate buffer, pH 7.1
* **B Buffer:** 1.4M NaCl, in 20mM phosphate buffer, pH 7.1
* **C Buffer:** 2M guanidine HCl in 20mM phosphate buffer, pH 7.1
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**Procedure:**

1. A column of Affi-Gel blue gel, 50-100 mesh, was prepared with a total bed volume of 5 ml of gel per ml of serum to be processed.
2. The column was pre-washed with 2 bed volumes of buffer A.
3. The serum sample was equilibrated in buffer A by dialyzing overnight.
4. The equilibrated serum sample was applied onto the pre-washed column.
5. After sample application, the column was washed with 2 bed volumes of buffer A. The flow rate was maintained at 0.5 ml per min. The effluent from this step was collected in 1.5 ml microcentrifuge tubes and contained the serum proteins minus most of the albumin.
6. Bound albumin was eluted with buffer B.
7. The column was regenerated with 2 bed volumes of buffer C.

20 μl of each fraction was run in SDS polyacrylamide gel (the principle is discussed in the section on SDS-Polyacrylamide gel electrophoresis) to check for the purity. The fractions, containing the protein of interest to the maximum extent but minimum amount of contaminant proteins, were pooled and concentrated. Concentration was carried out either by lyophilization or with the help of Centriplus YM-30 (Amicon, Millipore, USA). The concentrated proteins were dialyzed against the appropriate buffer (as mentioned earlier) before loading on to the next column for further purification.

**II. Sepharose-6B Size Exclusion/Gel Filtration Chromatography:** The separation of molecules on the basis of their molecular size and shape utilizes the molecular sieve properties of a variety of porous materials. The most commonly used of such materials are a group of polymeric organic compounds which possess a three-dimensional network of pores which confer gel properties upon them. The term gel filtration is used to describe separation of molecules of varying molecular size utilizing these gel materials.

The general principle of exclusion chromatography is quite simple. A column of gel particles is in equilibrium with a suitable solvent for the molecules...
to be separated. Large molecules which are completely excluded from the pores will pass through the interstitial spaces, while smaller molecules will be distributed between the solvent inside and outside the molecular sieve and will then pass through the column at a slower rate. Gel filtration is a simple and reliable chromatographic method for separating molecules according to size. Its versatility makes it generally applicable for purification of all classes of biological substances, including macromolecules not readily fractionated by other techniques.

Superose 6 is designed for high resolution chromatography. Superose 6 prep grade permits easy, efficient scale-up to preparative separations. Superose 6 prep grade is a cross-linked, agarose-based medium optimized for high performance gel filtration of biomolecules. The size and narrow distribution of the particles allow high flow rates, high efficiency and great capacity. Their gelling properties are attributed to hydrogen bonding of both inter- and intra-molecular type. Due to their hydrophilic nature and nearly complete absence of charged groups, agarose gels, like dextran gels, cause very little denaturation and adsorption of sensitive biochemical substances.

Buffer Formulations for gel filtration:

A Buffer: 50mM phosphate buffer, pH 7.4

Procedure:

1. To prepare the slurry, Sepharose 6B matrix was washed with Milli-Q water in a glass filter (porosity less than 10 mm) to remove all ethanol. The suspension must be homogeneous and free from aggregates and should be degassed before packing.

2. The column was packed at the temperature it was to be used i.e. 4°C. The packing reservoir was attached and the column was vertically mounted on a stand. The outlet tubing was closed. The column was wetted with eluent and the homogeneous gel suspension was poured down the inside wall of the column in one operation. The reservoir was
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gilled to the top with buffer A. The top of the packing reservoir was assembled and fastened and the outlet was opened.

3. Column equilibration: Before applying the sample, the column was equilibrated with two column volumes of buffer A. Longer equilibration may be needed with detergent solutions. Equilibration is not needed between runs with the same eluent.

4. The pooled fractions containing the protein of interest were equilibrated in buffer A by dialyzing overnight.

5. The equilibrated protein sample was then applied to the already equilibrated Superose 6 gel filtration column.

6. Washed the column with 2 bed volumes of buffer A. The eluent was collected in 1.5 ml microfuge tubes using a fraction collector. The flow rate was maintained at 1.0ml/5min and the eluent was collected in 1.5 ml eppendorfs.

Purification of the samples was checked by SDS-PAGE as before. The principle of the polyacrylamide gel electrophoresis is discussed in a later section. The fractions containing the protein of interest maximally were pooled and dialyzed against the desired buffer for further use.

III. Ion-Exchange Chromatography: Ion exchange chromatography is based on adsorption and reversible binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix. The pH value at which a biomolecule carries no net charge is called the isoelectric point (pI). When exposed to a pH below its pI, the biomolecule will carry a positive charge and will bind to a 'cation exchanger'. At pH above its pI the protein will carry a negative charge and will bind to an 'anion exchanger'. If the sample components are most stable below their pI, a cation exchanger should be used. If they are most stable above their pI, an anion exchanger is used. If stability is high over a wide pH range on both sides of pI, either type of ion exchanger can be used. The principle feature underlying this form of chromatography is the attraction between oppositely charged particles. Many biological materials, for example amino acids
and proteins, have ionisable groups and the fact that they may carry a net positive or negative charge can be utilized in separating mixtures of such compounds. The net charge exhibited by such compounds is dependent on their pK\textsubscript{a} and on the pH of the solution in accordance with the Henderson-Hasselbalch equation. Ion exchange chromatography is among the most precise and frequently used methods for the fractionation of biological substances. Biomolecules with even small differences in charge can be separated. Very high resolution is obtained by choosing the optimal ion exchanger and the optimal separation conditions.

For the present purification procedure DEAE Sephadex ion exchange (Amersham Biosciences) matrix was used. DEAE Sephadex is an anion exchanger.

Elution with stepwise ionic strength gradients involves the sequential use of the same buffer at different ionic strengths. It is technically simple and fast, and is suitable for syringe operation. It is often used for sample concentration and sample clean-up. Stepwise elution gives small peak volumes and the resolution depends on the difference in elution power between each step.

*Buffer Formulations for ion-exchange chromatography:*

*A Buffer: 0.05M Tris-HCl, pH 7.4*

*Gradient Buffers: Increasing NaCl gradient was prepared in Buffer A. The grades were 100mM, 115mM, 125mM, 135mM, 150mM and 200mM NaCl.*

Procedure:

1. The column matrix was equilibrated with buffer A.
2. The sample was adjusted to the chosen starting pH and ionic strength by dialyzing it overnight against various changes of buffer A.
3. The sample was applied at 5 ml/min and the eluate collected.
4. The column was washed with at least 5 column volumes of buffer A. The eluate was collected at a flow rate of 1.5ml/min in 1.5ml microfuge tubes.
5. The elution was started with the first step ionic strength buffer and the ionic strength was gradually increased, thus performing increasing
ionic gradient elution. The volumes required for stepwise elution depend on the operation. For the present purification procedure the volumes of the various grades used were as follows:

<table>
<thead>
<tr>
<th>Concentration (mM)</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>40</td>
</tr>
<tr>
<td>115</td>
<td>40</td>
</tr>
<tr>
<td>125</td>
<td>20</td>
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<tr>
<td>135</td>
<td>20</td>
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<tr>
<td>150</td>
<td>20</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
</tr>
</tbody>
</table>

The extent of purification achieved was again determined by SDS-PAGE. The fractions containing the protein of interest were pooled before loading on the phenyl sepharose column.

IV. **Hydrophobic Interaction Chromatography:** Hydrophobic interaction chromatography (HIC) is a versatile method for purification and separation of biomolecules based on the differences in their surface hydrophobicity. Proteins and peptides usually sequester hydrophobic amino acids in domains away from the surface of the molecule. However, many biomolecules considered to be hydrophilic have sufficient hydrophobic groups exposed to allow interaction with hydrophobic ligands attached to the chromatographic matrix. Compared to reversed phase chromatography, the density of the ligand on the matrix is much lower. This feature promotes high selectivity of HIC, while allowing mild elution conditions to help preserve biological activity. Hydrophobic interaction between a biomolecule and the matrix is enhanced by high ionic strength buffers. This makes HIC an ideal "next-step" for the purification of materials that have been precipitated with ammonium sulfate or eluted in high salt concentrations during ion exchange chromatography.

The type of immobilized ligand (alkyl/aryl), the degree of substitution and the type and concentration of salt and pH used during the adsorption stage affect the overall performance of a HIC medium. Phenyl Sepharose CL-4B is stable in
all commonly used aqueous buffers. It is based on cross-linked 4% agarose matrices with ligands coupled via stable ether linkages. It is estimated that as much as 40-50% of the accessible surface area of the proteins is non-polar. These areas are responsible for the binding of proteins to HIC adsorbents in the presence of moderate to high concentrations of salting-out salts. A step-wise decreasing gradient elution was carried out in the present case.

_Buffer Formulations for hydrophobic interaction chromatography:_

_A Buffer:_ 1.5M [(NH₄)₂SO₄] in 20mM phosphate buffer, pH 7.1

_Elution Buffer:_ Decreasing elution gradient from 1.5M to 0M [(NH₄)₂SO₄]

Procedure:

1. Column matrix phenyl sepharose CL-4B was equilibrated with buffer A.
2. The sample was adjusted to the chosen starting pH and ionic strength by dialyzing it overnight against various changes of buffer A.
3. The sample was applied at 5 ml/min and the eluate collected.
4. Washed with at least 5 column volumes of buffer A. The eluate was collected at the flow rate of 1.5ml/min in 1.5ml microfuge tubes.
5. The elution was started with the first step ionic strength buffer and then the ionic strength was gradually decreased, thus performing decreasing ionic gradient elution. The volumes required for stepwise elution depend on the operation. For the present purification procedure, 9-10ml of each grade was used for elution. The flow rate was maintained at 1.5ml/min.

GENERATION OF ANTIBODIES

Antibodies against the purified protein were commercially raised in rabbits as per standard protocol. Briefly, the pre-immune serum was collected before beginning the immunization protocol. Immunization schedule began by injecting 50μg of purified protein into the rabbit which was considered as day 1 of the immunization protocol. Two booster doses of 20μg each were given to the rabbit
on day 30 and day 45 of the protocol. Blood was withdrawn on day 55 and day 65 to collect a total of 25ml of serum. The immune serum was then affinity purified to get monospecific sera against the protein. The pre-immune serum was tested against the immunized sera and the working dilution (1:1000) standardized by Western blot analysis.

**SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

The universal method used today for analysis of proteins is electrophoresis in polyacrylamide gels. Microgram amounts of protein can be used to give information about a protein’s molecular weight, isoelectric point etc. SDS-gel electrophoresis is probably the most commonly used electrophoretic technique in protein chemistry. SDS (Sodium Dodecyl Sulphate) is an anionic detergent that has a polar sulphate group linked to a non polar aliphatic chain. It reacts with the proteins before electrophoresis. Most proteins bind about 1.4 gm of SDS per gram of protein and the resulting denatured protein complex has been thought to approximate to a rod like structure of uniform negative charge. With the exception of a few structural proteins, protein-SDS complexes are soluble. Under the influence of an electrical field these complexes migrate through a polyacrylamide gel towards the anode, generally at a rate inversely proportional to the logarithm of the molecular weight of an unknown protein when run together with proteins of known mol wt.

There are a number of systems for SDS-gel electrophoresis but the one developed by Laemmli (1970) is most widely used, especially for slab gels. The gel is cast in two parts. A ‘running gel’ or ‘separating gel’ is polymerized first. The buffer is Tris-HCl, pH 8.8 and contains SDS 0.1% w/v. The concentration of the acrylamide and ‘bis’ can be varied from about 5% T for electrophoresis of proteins of 200kDa to 20% T for low mol wt proteins, say of 5kDa. Once this gel has polymerized, a ‘stacking gel’ is polymerized on top of it. This is usually 2 cm deep and wells are formed in it with a suitable template. The buffer of the stacking gel is Tris-HCl, pH 6.8, SDS 0.1% w/v. The acrylamide concentration is usually between 3-5% T and should be as low as possible to eliminate any sieving effect.
The reservoir buffer is Tris-Glycine, pH 8.3, 0.1% w/v SDS. The buffer should not contain any other ions.

Proteins were dissolved in loading buffer, containing Tris-HCl pH 6.8, 10% glycerol to make it dense, 2-3% SDS and 0.01% w/v bromophenol blue (BB). BB is used as a ‘tracking dye’ to track the distance the proteins have migrated from their starting point. Such a dye runs at or near the front of electrophoresis and can be used to monitor the progression of the run and possibly show up any erratic migration. If the disulfide bonds of the proteins are to be reduced, β-mercaptoethanol is added to 5% v/v. It is important to make sure that SDS has fully reacted with the protein and this can be done by heating to 100°C for three min or 37°C for one hour. Proteins that contain disulfide bonds will not be fully denatured by the SDS unless they are fully reduced.

Once the samples were loaded, electrophoresis was carried out with the cathode as the top electrode since SDS-protein complexes are all negatively charged. The run was carried out in two hours at 80 mV constant voltage when the sample was in the stacking gel and at 120 mV once the dye-front moves a little distance in the resolving gel. The run was stopped once the tracking dye bromophenol blue reached the bottom of the gel. At the end of the run, the gel was removed and the proteins detected by staining. The ‘stacking’ system is very useful, for it leads to sharp and straight bands. The principle of stacking is isotachophoresis. The band-sharpening effect relies on the fact that the negatively charged glycinate ions (in the reservoir buffer) have a lower electrophoretic mobility than the protein-SDS complexes, which in turn have lower mobility than the Cl ions of the loading buffers and the stacking gel. When the current is turned on, all the ionic species have to migrate at the same speed otherwise there would be a break in the electrical circuit. The glycinate ions can only move at the same speed as the Cl ions if they are in a region of higher field strength. Field strength is inversely proportional to conductivity which is proportional to concentration. The result is that the three species of interest adjust their concentrations so that [Cl]>[protein-SDS]>[glycine]. There is only a small quantity of protein-SDS complexes so they concentrate in a very tight band between glycinate and Cl ion boundaries. Once the glycinate reaches the running gel it becomes more fully
ionized in the higher pH environment and its mobility increases. Thus the interface between the glycinate and the Cl⁻ ions leaves behind the protein-SDS complexes which are left to electrophorese at their own rates.

**VISUALIZATION OF THE GEL**

Once the proteins were separated by SDS-PAGE, they were detected on gels by staining with the sulphated trimethylamine dye Coomassie Brilliant Blue R250 (CBB). Staining was carried out using 0.02% (w/v) CBB in methanol:water:glacial acetic acid (45:45:10, by vol). This acid-methanol mixture acts as a denaturant to precipitate or fix the protein in the gel, which prevents the protein from being washed away whilst being stained. Staining of most gels was accomplished in about 2 hours and usually destained overnight. The latter was achieved by gentle agitation of the stained gel in the same acid-methanol solution but in the absence of the dye. The CBB stain is a highly sensitive stain and detects about 0.1μg (100ng) of protein.

Although the CBB stain is sensitive, for detection of minute quantities of impurities during the purification procedure, silver staining protocol was followed (*PROTOCOL 2*). Silver stains are based either on the techniques developed for histology or on methods based on the photographic process. In either case, silver ions (Ag⁺) are reduced to metallic silver on the protein, where the silver is deposited to give a black or brown band. The silver stain is at least 100 times more sensitive than CBB, detecting proteins down to 0.1ng amounts.

**WESTERN BLOTTING**

Electrophoretic transfer of proteins from an acrylamide slab gel (10% SDS-PAGE) to a transfer membrane (nitrocellulose) may be performed using either a tank system or a so-called semi-dry apparatus. A tank system consists of a rectangular buffer tank of about one litre capacity. The transfer ‘sandwich’, consisting of the slab gel juxtaposed to the transfer membrane, is firmly held between two perforated Plexiglas sheets, and is held in a vertical position between platinum wire electrodes. In the semi-dry apparatus (Biorad) the sandwich was
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placed directly between two flat carbon electrode plates, as per the instruction manual.

Once transferred on to nitrocellulose, the separated proteins can be examined further (**PROTOCOL 3**). This involves probing the blot, using an antibody to detect a specific protein (200kDa protein of interest in the present study). The blot was first incubated in a protein solution, 10% (w/v) bovine serum albumin (BSA) or 5% (w/v) non-fat dried milk, which blocked all remaining hydrophobic binding sites on the nitrocellulose sheet. The blot was then incubated in diluted antiserum (primary Ab 1:1000) directed against the 200kDa protein. This IgG (immunoglobulin) molecule binds to the blot if it detects its Ag, thus identifying the protein of interest. In order to visualize this interaction the blot was then incubated further in a solution of HRPO labeled anti-rabbit secondary Ab (1:10,000, Chemicon), which was directed against the IgG of the species that provided the primary Ab. Since the primary Ab was raised in rabbit, the secondary Ab used was anti-rabbit IgG. Following treatment with HRPO (horse radish peroxidase) labeled secondary Ab, the blot was incubated in the substrate solution containing diaminobenzidine (DAB). HRPO oxidized DAB in the presence of H₂O₂ into an insoluble coloured product that precipitated onto the nitrocellulose. The presence of a colored band therefore indicated the position of the protein of interest. An alternative approach to detect HRPO was to use the method of enhanced chemiluminescence (ECL). In the presence of H₂O₂ and the chemiluminescent substrate luminal, HRPO oxidized the luminal with concomitant production of light, the intensity of which increases 1000-fold by the presence of a chemical enhancer. The light emission was detected by exposing the blot to a photographic film.

**FUNCTIONAL ANALYSIS OF THE PROTEIN USING ENZYMATIC ASSAYS**

**Trypsin Assay:** The procedure is based on a method described by Erlanger et al. (1961) for determining trypsin activity with benzoyl-alpha-para-nitro-anilide (BAPNA) as a substrate. 217 μl of a 0.05M Tris buffer, pH 7.4, containing 0.02 M
CaCl$_2$ was thoroughly mixed with 1µl trypsin (1µg). 5-10 sec later, 0.33µg/0.5µg of trypsin inhibitor or 2.5µg/5µg of purified protein (25µl/50µl) was added and the mixture vortexed thoroughly and incubated for 10 min at 30°C. A further volume of 533µl of BAPNA solution was added, after which the sample was placed in a water bath and incubated at 30°C for 10min. A blank without trypsin and a control with trypsin alone (without any inhibitor) were incubated simultaneously. The reaction was stopped after 10min by addition of 266µl of 30% acetic acid. The absorbance of the sample at 410nm was read.

**Chymotrypsin Assay:** Chymotrypsin inhibitory property of the 200kDa protein was checked using N-acetyl-L-tyrosine-ethyl-ester (ATEE) as a substrate. 0.04 X 10$^{-6}$ mols (0.04µmols) of chymotrypsin were incubated for 3 min at 25°C with 0.03 X 10$^{-6}$ mols (0.03µmols) of purified protein or 0.04 X 10$^{-6}$ mol (0.04µmol) of chymotrypsin inhibitor. The reaction was started by the addition of 1ml of ATEE substrate in 0.067M potassium phosphate buffer, pH 7.0 and the hydrolysis rate was followed spectrophotometrically for 10 min at 237 nm.

**PREPARATION OF THE BRAIN CYTOSOLIC PROTEINS**

Cytosolic proteins were isolated from the brains using standard protocol (Krapfenbauer et al. 2001) (PROTOCOL 4). Rats (FMC, REMSD, LPC, REC) were sacrificed by cervical dislocation and the brains (including cerebral cortex and hippocampus) removed by decapitation. The whole brain tissue (1.0 g) was suspended in and washed thoroughly with ice cold homogenizing buffer (Sucrose-Tris buffer containing 0.32M sucrose, 12mM Tris, pH 7.4) to remove blood. The suspension was then homogenized in 10 volumes of homogenizing buffer in the Potter-Elvehjem homogenizer (Takashima Industry, Tokyo, Japan) with a Teflon pestle. The homogenate was spun at 800g (5000rpm) in a refrigerated centrifuge (SM-24 rotor of Sorvall RC-5B) for 10min to remove nuclei and undissolved protein. The supernatant of this step was then spun at 10,000g (~11,500rpm using the SM-24 rotor of Sorvall RC-5B) for 15min in order to remove the mitochondrial proteins. The supernatant of this centrifugation step was further centrifuged at 100,000g (~35,000rpm in the Beckman L 780 ultracentrifuge) for 1
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hour. The supernatant of the last step contained mainly cytosolic proteins and was used for further analysis.

**Protein estimation:**

**Principle:** Protein estimation was carried out by the method of Lowry et al. (1951). Protein reacts with Folin’s reagent (solution of tungstate and sodium molybdate in phosphoric and hydrochloric acid) to give a coloured complex. The colour so developed is due to the reaction of copper with protein in an alkaline medium. Alkaline copper sulphate reacts with compounds containing two or more peptide bonds to give a violet coloured complex. The intensity of the colour is proportional to the number of peptides present in the protein. Colour is also formed due to the reduction of phosphomolybdate by tyrosine and tryptophan present in the protein.

**Procedure:** The reaction mixture contained a suitable aliquot of the sample whose protein concentration was to be estimated and double distilled water was added to make up the volume to 500μl. 5ml of freshly prepared Lowry’s reagent was added. The mixture was vortexed and incubated at room temperature for 10min. 500μl of 1N Folin’s reagent was added, vortexed immediately and incubated for 30 min at room temperature. The colour developed was read at 700nm in a Shimadzu UV 160 spectrophotometer. Bovine Serum Albumin (BSA: 1mg/ml) was used as a standard.

Equal amounts of protein (40μg) from all the four groups of rats were separated on a 10% SDS-PAGE gel and then transferred onto an immunoblot for detection of A113 by using the monospecific antisera raised in rabbits. The principle and procedure of immunodetection of the protein has already been discussed previously.

**IMMUNOHISTOCHEMISTRY**

The animals (male wistar rats) were deeply anaesthetized with sodium pentobarbital (45 mg/kg body wt i.p.; Sigma) at the end of the experiment. They were then perfused transcardially, by the gravity method, with 0.9% phosphate-buffered saline (PBS: 100ml) followed by 200ml of phosphate-buffered 4%
paraformaldehyde (pH 7.0) as fixative. Fixatives preserve cells and tissues in a reproducible and life-like manner. Furthermore, they stabilize cells and tissues thereby protecting them from the rigors of processing and staining techniques. Fixatives may work either by formation of cross-linkages (e.g. aldehydes such as formalin) or protein denaturation by coagulation (e.g. acetone and methanol) or a combination of the above. After perfusion, the cranium was opened and the brain removed. The brains were post-fixed overnight, cryoprotected in 30% sucrose and stored at 4°C till further use.

Coronal sections were cut at 40μm on a Leica CM 1900 cryostat. The brains were cut into chucks of brain region required and fixed on to the chuck holders using tissue embedding fluid (OCT compound: Optimal Cryopreserving Tissue compound). They were then placed inside the cryostat and allowed to cool until the whole embedding fluid was frozen into a solid mass along with the tissue. One chuck holder was attached to the block holder of the cryostat arm (maintained at -15°C) and aligned such that the tissue was parallel to the knife (maintained at -30°C). A ribbon of sections was obtained which were subsequently placed in 5ml beakers containing PBS. Immunohistochemistry gives better results when done on free floating sections.

**Principle:** The word histochemistry is used to describe the accurate localization of tissue components *in-situ* by a chemical reaction. When this is done using antibodies against the enzyme or its product, the technique is known as immunohistochemistry. Immunohistochemistry is widely used in experimental neurobiology to characterize neurons according to the specific neurochemical. Antibodies raised against the specific neurochemical get bound to the antigenic sites on the neurons or at the terminals. The site of antigen-antibody (Ag-Ab) binding is then localized in the tissue using several techniques. In the direct method, the antigen-antibody complex is visualized by a fluorescent molecule tagged to the antibody that can be detected by a fluorescent microscope. However, this technique works better in single cell suspensions, while such a signal is too weak to be detected in a tissue. In the indirect method, the antibody (primary Ab) gets bound to the antigenic site in the tissue. The tissue is then incubated with the secondary Ab directed against the primary Ab. This binds to the Ag-primary Ab
complex on the tissue. The secondary Ab is conjugated to a marker like a fluorescent molecule and directly viewed under the microscope. Alternately, the secondary Ab can be conjugated to horseradish peroxidase (HRPO) which can be visualized under the microscope after staining with diaminobenzidine (DAB).

**Procedure:** The endogenous peroxidases present in the tissue were blocked by incubating each set of coronal brain sections with 4% hydrogen peroxide ($\text{H}_2\text{O}_2$) in 90% methanol for 1 to 1.5 hours. The next step involved the reduction of non-specific binding of the primary Ab to sites other than the Ag binding site. This was done by pre-treating the sections with normal serum from the species in which the secondary Ab was raised. In the present study normal goat serum (10% NGS) was used at a dilution of 1:200. The Na-K ATPase molecules in histological sections of FMC, REMSD, LPC and REC rats were immunostained with monoclonal antibody, mAb 9A7 against the $\alpha_1$ sub-unit of Na-K-ATPase (gift from Prof. Masami Takahashi, School of Medicine, Kitasato University, Japan) (1:350 in 0.1M PBS containing 5% normal goat serum and 0.5% Triton X-100). The incubation was carried out for 2h at room temperature (*PROTOCOL 5*).

Alternatively, sections were also incubated for 2 days at 4°C in the monospecific primary antibody (raised against the purified 200kDa protein; 1:2000) (*PROTOCOL 6*). The dilutions of the primary and secondary Ab were done in the serum of the species in which it was raised (NGS in this case). 0.5% Triton X-100 was used while preparing the dilutions of the Abs, since it is a detergent that helps in permeabilizing the cell membrane and penetration of the Ab in the tissue. After washing with PBS, the sections were incubated with the biotinylated secondary antibody for 18 hours. The secondary Ab used was against rabbit and raised in goat (1:1000 dilution; Vector Laboratories, Burlingame, CA). Consequently, the free floating sections were reacted with avidin-biotin-peroxidase complex for 2 hours after washing them with PBS. Vectorstain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used in this study. It contained avidin DH and biotinylated HRPO reagents. The combination of these two formed a three dimensional array that had at least one biotin binding site. The ABC complex bound to the biotinylated secondary Ab. The complex could be detected using a variety of chromogens that bind to peroxidases. The chromogen used was DAB.
with $\text{H}_2\text{O}_2$ as the substrate reagent. The reaction product resulted in a brown precipitate at the antigenic site of the tissue.

Immunostained sections were then placed over slides which had previously been subbed in gelatin. When wet tissue is placed over subbed slides, they become sticky and after drying, the gelatin acts as a good adhesive for holding the sections to the slides. Stained sections were mounted in a resinous medium, Distrene Plasticiser Xylene (DPX), which forms a transparent layer of suitable refractive index between the slide and the coverslip. Resinous media dissolved in organic solvents such as xylene. Sections were passed through a grade of alcohol (30%, 50%, 70%, 90%, 100%) rather than direct 100% to avoid tissue shrinkage with abrupt contact with absolute alcohol. Finally the slides were dipped in xylene and mounted with a coverslip.

**Brain areas used for assessment:**

Atleast two distinct nuclear groups have been implicated for the generation of REM sleep. They are the cholinergic neurons in laterodorsal tegmentum and pedunculopontine tegmentum (LDT/PPT) and the noradrenergic neurons in locus coeruleus (LC) in the dorsolateral pons. Thus, in the present study, the brain areas selected for studying the effects of REM sleep deprivation were LDT/PPT, LC, medial preoptic area (mPOA) and lateral septum (Fig VIII) according to the atlas of rat brain by Paxinos and Watson (1997). The medial preoptic area is an important nuclei in the basal forebrain that is involved in sleep and

![Fig VIII](image)
thermoregulation. It is a sleep inducing area not directly related to REM sleep. This area was chosen to see if REM sleep deprivation affected an area that was involved in regulating NREM sleep. Lateral septum has no role to play in sleep-wakefulness. This area was chosen to act as a control area to see if the effect of REM sleep deprivation was restricted to sleep/REM sleep related areas or generalized throughout the brain.

**Data acquisition and analysis:**

Immunostained sections were seen under Nikon E400 microscope (magnification 400X). The magnified views of the neurons were captured in a computer with the help of a CCD camera (JVC, Japan) fitted onto the microscope (Fig IX) using the image grabber software (Chameleon, Univision Technologies, Burlington, USA). For the estimation of the concentration of Na-K ATPase and A113, the software Scion Image (Scion Corporation, USA) was used for the densitometric analysis. In these studies, individual neurons were outlined and the density of colour per unit area (after background subtraction) of the individual neuronal soma in LC, LDT/PPT, mPOA and lateral septum was proportional to the concentration of the protein molecules. Such estimations were done bilaterally in every third section. On an average there were 100 neurons estimated in every section and 10-12 such sections were studied in each group.

**IN VITRO EXPERIMENTS IN HEPATIC CELL LINES**

HepG2 and BRL3A are human and rat hepatic cell lines respectively. These cell lines were procured from NCCS (National Centre for Cell Science), Pune and maintained in the cell culture facility in the laboratory. The cells were maintained in a medium containing 90% Dulbecco's Modified Eagle Medium (DMEM) supplemented with pyruvate, sodium bicarbonate and sodium pyruvate and 10% fetal bovine serum (FBS). The cells were maintained and subcultured in 25cc flasks and maintained in a CO₂ incubator (Thermo Forma) supplied constantly with 5% CO₂. The temperature was sustained at 37°C and the humidity was maintained inside the incubator.
FIG IX SET-UP FOR DATA ANALYSIS
**Subculturing Protocol:**

The culture medium in which the cells had been growing was removed and discarded. The cell layer was briefly rinsed with PBS to remove all traces of debris. A volume of 0.5 ml of trypsin-EDTA solution was added to the flask and the cells were observed under an inverted microscope until the cell layer was dispersed (usually within 2 to 5 minutes). (Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal). Added 5.0 ml of complete growth medium and cells were aspirated by gently pipetting. Appropriate aliquots of the cell suspension were added to new culture vessels and these subcultures were incubated at 37°C. A subcultivation ratio of 1:4 to 1:6 was usually carried out with medium renewal twice a week.

Since the protein of interest (AII3) is a secretory glycoprotein, it should be secreted by the growing cells in the medium. The cells were plated on a culture dish. When the cells were 60% confluent they were transferred into a serum free medium. Subsequently, 200μl of the medium was aspirated at various time points viz., 1h, 3h, 5h, 8h, 12h, 24h, 36h and 48h. Equal volume of the aspirated sample (20μl) was separated on 10% SDS-PAGE and silver stained to check for the secretory protein profile. Aliquots of 8th h, 12th h, 36th h and 48th h from HepG2 and BRL3A cell lines were immunoblotted to check for the change in the level of AII3 using the monospecific antibodies against the protein.

**Dose response effect of NE:**

A dose response of NE was done to see the role of NE on the modulation of AII3 secreted by HepG2 cells in culture. Cells were plated on a culture dish. They were transferred into serum free medium when they attained 60% confluency. Various doses of norepinephrine were added into the medium viz., 0μM, 0.5μM, 1.0μM, 2.0μM, 5.0μM, 10μM, 20μM, 50μM, 100μM, 200μM, 250μM and 500μM. 200μl of medium was aspirated at 8th h, 24th h and 36th h after NE treatment. Equal volume of the medium (20μl) was then separated on a 10% SDS-PAGE gel and visualized by silver staining.
IN VIVO INTRA-PERITONEAL INJECTIONS

Prazosin:

The α₁-adrenergic antagonist, Prazosin (Pz), was injected intraperitoneally (i.p.) (4 mg/Kg body wt) in free moving control (FMC) rats (n=3) and 6 days REM sleep deprived (REMSD) rats (n=3). Blood was collected and the respective sera isolated at different time points from both the FMC and REMSD rats at (a) start of deprivation; (b) after 4 days of deprivation; (c) after 6 days of deprivation and (d) 6hrs post-prazosin injection.

Propranolol:

The β-adrenergic antagonist, Propranolol (Pr), was injected intraperitoneally (i.p.) (10 mg/Kg body wt) in free moving control (FMC) rats (n=3) and 6 days REM sleep deprived (REMSD) rats (n=3). Blood was collected and the respective sera isolated at different time points from both the FMC and REMSD rats at (a) start of deprivation; (b) after 4 days of deprivation; (c) after 6 days of deprivation; (d) 3hrs post-propranolol injection and (e) 6hrs post-propranolol injection.

Clonidine:

The α₂-adrenergic agonist, Clonidine (Cl), was injected intraperitoneally (i.p.) (0.1 mg/Kg body wt) in free moving control (FMC) rats (n=3) and 6 days REM sleep deprived (REMSD) rats (n=3). Blood was collected and the respective sera isolated at different time points from both the FMC and REMSD rats at (a) start of deprivation; (b) after 4 days of deprivation; (c) after 6 days of deprivation; (d) 3hrs post-clonidine injection and (e) 6hrs post-clonidine injection.

NORTHERN HYBRIDIZATION

Northern hybridization is used to estimate the amount and size of RNAs transcribed from eukaryotic genes and to estimate their abundance. No other method is capable of obtaining these pieces of information simultaneously from a
large number of RNA preparations. Northern analysis is therefore fundamental to studies of gene expression in eukaryotic cells. Northern hybridization became a part of the standard repertoire of molecular biology almost immediately after the first descriptions of the method were published (Alwine et al. 1977, 1979). Although many variations and improvements have been published during the succeeding 20 years, the basic steps in northern analysis remain unchanged:

- isolation of intact mRNAs
- separation of RNA according to size through a denaturing agarose gel
- transfer of the RNA to a solid support in a way that preserves its topological distribution within the gel
- fixation of the RNA to the solid matrix
- hybridization of the immobilized RNA to probes complementary to the sequence of interest
- removal of probe molecules that are nonspecifically bound to the solid support
- detection, capture and analysis of an image of the specifically bound probe molecules.

**Isolation of total RNA:**

A typical mammalian cell contains $\sim 10^5 \mu$g of RNA, 80-85% of which is ribosomal RNA, rRNA (chiefly the 28S, 18S, 5.8S and 5S species). Most of the remaining 15-20% consists of a variety of low-mol wt species (e.g. transfer RNA, tRNA and small nuclear RNA, snRNA). Messenger RNA (mRNA), which makes up between 1% and 5% of the total cellular RNA, is heterogenous in both size (from a few hundred bases to many kilobases in length) and sequence. This heterogenous population of molecules collectively encodes virtually all of the polypeptides synthesized by the cell. The key to successful purification of intact RNA from cells and tissues is speed. Cellular RNases should be inactivated as quickly as possible at the very first stage in the extraction process. Once the endogenous RNases have been destroyed, the immediate threat to the integrity of the RNA is greatly reduced, and purification can proceed at a more graceful pace.
Principle: The protocol followed for isolation of total RNA from the liver tissues was a variation of the single step method for RNA extraction (Chomczynski 1993). The method involves lysis of cells with a monophasic solution of guanidine isothiocyanate and phenol. Addition of chloroform generates a second (organic) phase into which DNA and proteins are extracted, leaving RNA in the aqueous supernatant. The DNA and the protein can be isolated from the organic phase by sequential precipitation with ethanol and isopropanol respectively. The RNA precipitated from the aqueous phase with isopropanol can be further purified by chromatography on oligo(dT)-cellulose columns and used for northern blot hybridization, reverse transcription or RT-PCRs.

The yield of total RNA depends on the tissue or cell source, however it is generally 4-7μg/mg starting tissue or 5-10μg/10^6 cells. The A_{260}/A_{280} ratio of the extracted RNA is generally 1.8-2.0.

Procedure: The monophasic lysis reagent used in the present study for total RNA isolation was the TRI-reagent (Sigma, USA). It is a quick and convenient reagent for simultaneous isolation of RNA, DNA and protein.

The first step involved sample preparation from the tissues (PROTOCOL 7). The tissue used in the present study was liver taken from the FMC, REM sleep deprived (6 days), LPC and REC rats. For this, 50mg of liver tissue from the respective animal was homogenized in TRI-reagent (1ml per 50-100mg of tissue) using a pre chilled mortar and pestle washed in DEPC treated Milli-Q water. 200μl of chloroform was added per ml of TRI reagent used. The samples were covered tightly, shaken vigorously for 15sec and allowed to stand for 15min at room temperature. The resulting mixture was centrifuged at 12,000g for 15min at 4°C. Centrifugation separated the mixture into three phases: a red organic phase (containing protein), an interphase (containing DNA), and a colourless upper aqueous phase (containing RNA). (Note: The chloroform used for phase separation should not contain isoamyl alcohol or other additives.)

The second step was RNA isolation. The aqueous phase was transferred to a fresh eppendorf and 500μl of isopropanol was added per ml of TRI reagent used in the sample preparation and mixed. The sample was allowed to stand for 5-10min at room temperature. Centrifuged at 12,000g for 10min at 4°C. The RNA
precipitate formed a pellet on the side and bottom of the eppendorf. The supernatant was removed and RNA pellet was washed by adding 1ml (minimum) of 75% ethanol per 1ml of TRI reagent used in sample preparation. The sample was vortexed and then centrifuged at 7500g for 5min at 4°C. (Note: Samples can be stored in 75% ethanol at 4°C for atleast 1 week and upto 1 year at -20°C). The RNA pellet was air-dried for 5-10min. (Note: The RNA pellet was not dried completely, as this greatly decreases its solubility). An appropriate volume of DEPC treated water was added to the RNA pellet. To facilitate dissolution of the RNA, mixed by repeated pipetting with a micropipette at 55-60°C for 10-15min. (Note: Final preparation of RNA should have $A_{260}/A_{280}$ ratio of 1.7).

Separation of RNA according to size: Electrophoresis of RNA through agarose gels containing formaldehyde:

Samples of RNA may be denatured by treatment with formamide and separated by electrophoresis through agarose gels containing formaldehyde. In this method RNA is fractionated by electrophoresis through an agarose gel containing 2.2M formaldehyde (Lehrach et al. 1977; Goldberg 1980; Seed 1982; Rosen et al. 1990).

Principle: Formaldehyde forms unstable Schiff bases with the single imino group of guanine residues. These adducts maintain RNA in the denatured state by preventing intrastrand Watson-Crick base pairing. Because the Schiff bases are unstable and easily removed by dilution, RNA can be maintained in the denatured state only when formaldehyde is present in the buffer or gel.

Procedure: Set up the denaturation reaction. In sterile microfuge tubes mix:

- RNA (upto 20μg) 2.0μl
- 10X MOPS electrophoresis buffer 2.0μl
- Formaldehyde 4.0μl
- Formamide 10.0μl
- Ethidium Bromide (200μg/ml) 1.0μl

The microfuge tubes were closed and the RNA solutions in it were incubated for 60min at 55°C. (Alternatively, RNA solutions can be incubated at 85°C for 10min.). The samples were chilled for 10min in ice water, and then
centrifuged for 5 sec. Then 2 μl of 10X formaldehyde gel loading buffer was added to each sample and the tubes were returned to the ice bucket. 1% agarose/2.2M formaldehyde gel in a horizontal electrophoresis box was polymerized. Sufficient 1X MOPS electrophoresis buffer was added to cover the gel to a depth of ~1 mm. The gel was run for 5 min at 5 V/cm and then loaded the RNA samples into the wells of the gel. The gel was run submerged in 1X MOPS electrophoresis buffer at 4-5 V/cm until the bromophenol blue migrated ~8 cm (4-5 hours). The RNA was visualized by placing the gel on a piece of Saran Wrap on a UV illuminator. Once the integrity of the isolated RNA was confirmed, immobilization of RNA onto a solid support by upward capillary transfer was proceeded.

Transfer and Fixation of denatured RNA to membranes:
In most cases, fractionation of RNA by agarose gel electrophoresis is but a prelude to hybridization of the fractionated population to specific labeled probes that detect particular target mRNAs. RNA is first transferred from an agarose gel to a two-dimensional support, usually a nylon membrane.

**Principle:** Transfer to uncharged nylon membranes is carried out at neutral pH, usually in 10X or 20X SSC (Salt of Sodium Citrate). The RNA is then covalently linked to the matrix by the traditional method by baking under vacuum for 2 hours, by heating in a microwave oven for 2-3 min (Angeletti et al. 1995), or by exposing the nylon membrane to UV irradiation at 254/312 nm.

**Procedure:** Unused areas of the gel were trimmed using a sharp scalpel. Cut along the slot line to allow the top of the trimmed gel to be aligned with the top of the membrane during transfer. A piece of thick blotting paper was placed on a sheet of glass plate, to form a support that was longer and wider than the trimmed gel. It was made sure that the ends of the blotting paper droop over the edges of the plate. The support was placed inside a large baking dish and the dish was filled with 20X SSC buffer. The appropriately cut nylon membrane was immersed in 20X SSC for 5 min. The gel was carefully placed on the support in an inverted position so that it was centered on the wet blotting paper. The top of the gel was wetted with the appropriate transfer buffer. The wet nylon membrane was placed on top of the gel so that the cut corners were aligned. Two pieces of thick blotting
paper (cut to exactly the same as size as the gel) were wetted in the appropriate transfer buffer and placed on top of the wet nylon membrane. Any air bubbles were smoothened out with a glass rod. A stack of paper towels (5-8cm high) just smaller than the blotting papers were cut and placed on the blotting papers. The glass plate was put on top of the stack and was weighed down with a 400-500gm weight. Upward transfer of RNA was allowed to occur for 18 hrs. The capillary transfer system was dismantled and the positions of the slots on the membrane were marked with a pencil through the gel. The membrane was placed on a piece of dry blotting paper and irradiated at 254nm for 2min at 1.5J/cm².

**Northern Hybridization:**

RNA samples that have been transferred and fixed to a membrane may be hybridized with a specific probe to locate the RNA species of interest. After treating the membranes with blocking agents that suppress nonspecific absorption of the probe, the membrane is incubated under conditions that favour hybridization of the labeled probe to the immobilized target RNA. The membrane is then washed extensively to remove adventitiously bound probe and finally manipulated to yield an image of the distribution of the tightly bound probe on the membrane.

**Procedure:** The Nylon membrane was incubated for 3-4 hours at 60°C in 10-12ml of pre-hybridization buffer solution. The probe was radiolabeled as per the instructions given in the manual of the labeling kit (NEBlot Kit, New England Biolab, USA). The $^{32}$P-labeled double stranded DNA was denatured by heating for 5min at 100°C and chilled rapidly in ice water. The denatured or single stranded radiolabeled probe was added directly to the pre-hybridization solution. Incubation was continued for 12-16 hrs at the appropriate temperature (60°C). After hybridization, the membrane was removed from the chamber and washed at room temp with 20ml of pre-warmed 4X SSC, 2% SDS. The box was placed on a platform shaker and the fluid gently agitated for 10min. The membrane was washed with 20ml of 2X SSC, 1% SDS, pre-warmed to 62°C. The fluid was gently agitated for 10min at room temp. The membrane was finally washed with 1X SSC, 1% SDS at room temp. The membrane was placed on Saran Wrap and an
autoradiograph was established by exposing the membrane for 24-48 hrs to X-ray film (Kodak XAR-5) at -80°C with an intensifying screen. Alternatively an image of the membrane was also obtained by scanning in a phosphorimager.

**Synthesis of Al13 probe:**

The probe used for detecting the Al13 mRNA was 0.6kb cDNA fragment synthesized by Polymerase Chain Reaction (PCR) reaction. The plasmid pRLalpha113/23J, containing the cDNA insert length of 4.6kb, codes for the gene product alpha-1-inhibitor III, group 1 (Al13A). Transformed E. Coli containing the vector pcDV1 carrying the insert pRLalpha113/23J (Catalogue no. 63100) were procured from American Type Culture Collection (ATCC), USA.

**Plasmid Isolation:**

**Principle:** Many methods have been developed to purify plasmids from bacteria. All of them basically involve three steps: growth of the bacterial culture, harvesting and lysis of the bacteria and finally purification of the plasmid. Lysis of bacteria and purification of plasmid is based on the use of three solutions. Solution I is the *Cell Suspension Solution* which contains Tris (pH 7.5), EDTA and glucose. The resuspended cell solutions' pH is raised to a basic level with Tris to help denature the DNA. EDTA stabilizes the cell membrane by binding the divalent cations of Mg$^{+2}$ and Ca$^{+2}$. Solution II is the *Cell Lysis Solution*. This solution contains SDS, an ionic detergent, which dissolves the phospholipids and protein components of the cell membrane. This lyses the membrane, releasing the cellular components. NaOH in the solution denatures the plasmid and chromosomal DNA into single strands. Solution III is the *Neutralization Buffer*. Potassium acetate from this solution forms an insoluble precipitate of SDS/lipid/protein complex and neutralizes the NaOH from the previous step. The DNAs renature at this neutral pH. Chromosomal DNA is trapped in the SDS/lipid/protein precipitate. The plasmid DNA renatures into its double stranded form, escapes being trapped in the precipitate and remains in solution (supernatant).
Procedure: Transformed *E. Coli* were transferred to a small starter culture in the LB (Luria Broth) medium, which was grown to late log phase at 37°C with vigorous shaking (220rpm). The plasmid contains the ampR (Ampicillin resistant) site. Bacteria were recovered by centrifugation at 12,000rpm for 5min at 4°C (*PROTOCOL 8*). The medium was aspirated, leaving the bacterial pellet as dry as possible. The bacterial pellet was resuspended in 100μl of "ice cold" solution I (cell suspension solution) by vigorous vortexing. Solution I contained 50mM glucose, 25mM Tris-HCl, pH 8.0 and 10mM EDTA, pH 8.0. 200μl of "freshly prepared" solution II (cell lysis solution) was added and mixed gently without vortexing. It was made sure that the entire surface of the tube came in contact with solution II. The microcentrifuge tube was then stored on ice. Solution II contained 0.2N NaOH and 1% SDS. Finally 150μl of "ice-cold" solution III was added. The tube was vortexed and stored on ice for 3-5 min. Centrifuged at 12,000rpm for 5min at 4°C. The supernatant was transferred to a fresh tube and equal volume of phenol:chloroform:isoamylalcohol (PCI) (25:24:1) was added. It was mixed by vortexing and the centrifuged at 12,000rpm for 5min at 4°C. The solution resolved into three layers. The upper clear aqueous layer was taken. 1/5th volume (one fifth volume of the aqueous layer volume) of 5M sodium acetate and 2 volume (twice the volume of the aqueous layer volume) of ethanol were added and kept at -20°C for 2-3 hours. Centrifuged at 12,000rpm for 10min at 4°C. The supernatant was discarded, taking care that the pellet was not disturbed. To this pellet added 1ml of 75% ethanol. Vortexed and centrifuged at 8000rpm for 5min at 4°C. The supernatant was discarded and the pellet was air dried. Dissolved the pellet in 20μl of autoclaved Milli Q water and 30μl of RNase A solution and kept for 1 hour at 37°C water bath to precipitate. RNase destroys the RNA from the cell contents when the cell is lysed. After the incubation period was over, 450μl of autoclaved Milli Q water was added such that the final volume became 500μl. To this, equal volume of PCI solution was added and dissolved by vortexing. Centrifugation was done at 12,000rpm for 10min at 4°C. Took the clear aqueous layer and added its 1/5th the volume of 5M sodium acetate and 2 volume of ethanol and kept it at -20°C overnight. Centrifuged at 12,000rpm for 10min at 4°C. The supernatant was discarded without disturbing the pellet. 1ml of 75%
ethanol was added and dissolved the pellet in it by vortexing. Centrifuged at 8000rpm for 5min a 4°C. The supernatant was discarded and the pellet air dried. The pellet was finally dissolved in 20μl of autoclaved Milli Q water and stored at -20°C till further use. The plasmid was quantified by reading the absorbance at A_{260}.

**Determination of authenticity of the plasmid:**

The authenticity of the plasmid was checked by performing a restriction digest of the plasmid. The purified plasmid was digested with EcoRI and HindIII at their respective restriction sites. The digested products were then run on 1% agarose gel to confirm the sizes of the restriction digest using appropriate mol wt markers.

**Synthesis of the probe:**

The entire cDNA sequence of the 4.6kb insert plasmid pRLalpha113/23J is known. 0.6kb region of the plasmid was amplified using PCR (Polymerase Chain Reaction) and then eluted from the gel using the Genelute Kit (Sigma, USA). The forward and reverse primers used for PCR were custom made by Sigma Genosys. The following are the 5'–3' sequence of the primers used:

Forward primer sequence (23nt): 5’-CATTGAGGATCCGAAAATGAACC-3’
Reverse primer sequence (22nt): 5’-GAATGGGATCCCGTGTCTGAAG-3’

The amplified product was 578 nt in length and lied in the region from the nucleotide number 580 to nucleotide number 1158 of the plasmid insert.
PROTOCOL 1
Protocol for activation of dialysis tubing

1. The dialysis tubing was soaked in 500ml of NaHCO₃/EDTA buffer and boiled for 5 min.

2. Allowed the solution to cool and discard it. Rinsed the dialysis tubing thoroughly with DW.

3. Again dip the tubing in 500ml of warm NaHCO₃/EDTA buffer. Boiled for 5 min.

4. Allowed the solution to cool and discard it. Rinsed the dialysis tubing thoroughly with DW.

5. Finally boiled the dialysis tubing in DW for 5 min before using it.

6. The activated dialysis tubing was stored in 0.1% sodium azide in DW.
PROTOCOL 2
Protocol for silver staining

1. The gel was fixed in 40% ethanol, 12% acetic acid, 25μl formaldehyde for 2-3 hrs with changes.
2. Washed thrice for 20min each in 50% ethanol.
3. Pretreated with sodium-thio-sulfate solution (0.02g/100ml) for 1 min.
4. Rinsed with Milli Q water thrice for 20 sec.
5. Impregnated with 50ml of silver nitrate (0.1g/50ml) and formaldehyde (35.5μl/50ml) solution for 20min in the dark.
6. Rinsed with Milli Q water thrice for 20 sec each.
7. Developed with 50ml of Na₂CO₃ (6%), 0.2μg of sodium-thio-sulphate and 25μl of formaldehyde for 1-2min.
8. Rinsed with Milli Q water.
9. The developing reaction was stopped by incubating the gel for 10 min in 50ml of 40% ethanol and 12% acetic acid.
10. Finally the gel was washed for 20min in 50% methanol.
11. The gel was stored in DW.
PROTOCOL 3
Protocol for Western blot

1. Washed the nitrocellulose blot (on which the proteins separated by SDS-PAGE have been transferred) for 10min in a solution of 25mM TBS (Tris Buffered Saline), pH 7.6.

2. Blocking of the blot was carried out overnight at 4°C with 10-15 ml of 3% BSA solution prepared in TBS.

3. Incubated the blot in 10ml of primary Ab (1:1000) for 1hr at 37°C. Primary Ab was prepared in 0.3% BSA in TBST (0.05% Tween-20 in TBS).

4. The blot was washed four times 20min each with TBST.

5. The blot was incubated in 10ml of HRPO labeled anti-rabbit IgG secondary Ab (1:10000) for 1hr at 37°C. Secondary Ab was prepared in 0.3% BSA in TBST (0.05% Tween-20 in TBS).

6. Washed thrice for 20min each with TBST. Gave a final wash for 20min with TBS solution.

7. Visualization was done by incubating the blot with DAB solution (6mg DAB, 10μl H2O2 in 10ml of 100mM Tris-Cl, pH 7.6). Reactive protein band started appearing from 5min onwards.

8. The reaction was stopped by washing off the reagent with DW and the blot was stored at 4°C for photography.
PROTOCOL 4
Protocol for isolation of brain cytosolic proteins

Whole brain tissue (1g) →

Homogenized in 10ml of Tri-Sucrose homogenizing solution →

Centrifuged at 800g (5000rpm) for 10min →

Pellet (nuclei and undissolved material) →

Supernatant →

Centrifuged at 10,000g (11,500rpm) for 15min →

Pellet (mitochondrial proteins) →

Supernatant →

Centrifuged at 100,000g (35,000rpm) for 1h →

Pellet (Cytosolic Proteins) →

Supernatant
PROTOCOL 5
Protocol for immunostaining with anti Na-K ATPase Ab (mAb 9A7)

1. Free floating sections (40µm) were treated with 4% H₂O₂ in 10ml of 90% methanol for 1.5h in the dark.
2. Washed in PBS (3 x 10min).
3. Blocked in 10% NGS and 0.5% Triton X-100 in PBS for 2h at room temperature.
4. Incubated with primary Ab (1:350 dilution) in PBS with 5% NGS and 0.5% Triton X-100 for 2 days at 4°C.
5. Washed in PBS (5 x 10min).
6. Incubated with secondary Ab (1:3000 dilution) in PBS with 1% NGS for 18h (4°C).
7. Washed in PBS (3 x 10min).
8. Incubated with ABC (1:200 dilution) in PBS for 2h at room temperature.
9. Washed in PBS (3 x 10min) and then in distilled water.
10. Treated with DAB and H₂O₂ in DAB substrate (Tris) buffer for 3min.
11. Distilled water washed immunostained sections were taken on slides, dehydrated and mounted with DPX.
12. Brown coloured immuno positive neurons were visible on a yellow background.
PROTOCOL 6

Protocol for immunostaining with anti-A113 Ab

1. Free floating sections (40µm) were treated with 4% H₂O₂ in 10ml of 90% methanol for 1.5h in the dark.
2. Washed in PBS (3 x 10min).
3. Blocked in 10% NGS and 0.5% Triton X-100 in PBS for 2h at room temperature.
4. Incubated with primary Ab (1:1000 dilution) in PBS with 5% NGS and 0.5% Triton X-100 for 2 days at 4°C.
5. Washed in PBS (5 x 10min).
6. Incubated with secondary Ab (1:3000 dilution) in PBS with 1% NGS for 18h (4°C).
7. Washed in PBS (3 x 10min).
8. Incubated with ABC (1:200 dilution) in PBS for 2h at room temperature.
9. Washed in PBS (3 x 10min) and then in distilled water.
10. Treated with DAB and H₂O₂ in DAB substrate (Tris) buffer for 3min.
11. Distilled water washed immunostained sections were taken on slides, dehydrated and mounted with DPX.
12. Brown coloured immuno positive neurons were visible on a yellow background.
PROTOCOL 7
Protocol for mRNA isolation

Rat liver tissue (50-100mg)

Homogenized the tissue in 1ml of TRI reagent

Left at room temperature for 5min

Added 200μl of chloroform and vortexed vigorously for 15sec

 Stored the mix at room temperature for 15min

Centrifuge at 12,000rpm for 15min at 4°C

Aqueous phase (RNA)

Interphase

Phenol-Chloroform

Organic phase

Took the aqueous phase (~500-600μl) and put into fresh RNase free microfuge tubes

Added 500μl of isopropanolol

Shake gently and kept at room temperature for 10min

Centrifuge at 12,000rpm for 15min at 4°C

RNA gets precipitated

Removed the supernatant and washed the RNA pellet by vortexing with 1ml of 75% ethanol

Centrifuge at 12,000rpm for 15min at 4°C

Removed ethanol and air dried the RNA pellet for 5min

Solubilized the pellet with 100-200μl of RNase free DEPC treated water
PROTOCOL 8
Protocol for plasmid isolation

Transferred a single bacterial colony to 4 ml of LB medium. Incubated overnight at 37°C with vigorous shaking (220rpm)

- Centrifuge at 12,000rpm for 5min at 4°C

Removed the medium leaving the bacterial pellet as dry as possible

- Resuspended the bacterial pellet in 100μl of Sol I

- Added 200μl of ‘freshly prepared’ Sol II

- Added 150μl of ‘ice cold’ Sol III

- Centrifuge at 12,000rpm for 5min at 4°C

- Added equal volume of PCI and mixed by vortexing

- Centrifuge at 12,000rpm for 15min at 4°C

Aqueous phase

White milky layer

Yellow PhenolChloroform

Organic phase

Took the aqueous layer and added 1/5th its vol sod acetate (5M) and 2 vol of ethanol and kept at -20°C for 2-3 hours

- Centrifuge at 12,000rpm for 10min at 4°C

To the pellet added 1ml of 75% ethanol and vortexed

- Centrifuge at 8,000rpm for 5min at 4°C

Air dried the pellet and then added 20μl of RNase A sol

- Incubate at 4°C for 1 hour

Repeated PCI extraction

- Air dried the pellet

Dissolved in 20μl of autoclaved Milli Q water and stored at -20°C till further use
APPENDIX FOR BUFFER FORMULATIONS

Activation buffer for dialysis tubing:
16.8 gm NaHCO₃ and 2.02 gm of EDTA were dissolved in 800 ml of DW. Final volume was made to 1000 ml.

Buffers for column chromatography:
Stock Solutions:

0.2M NaH₂PO₄ : 7.8gm/250ml
0.2M Na₂HPO₄ : 14.19gm/500ml

20mM Working Phosphate Buffer, pH 7.1:
67 ml of Na₂HPO₄ was mixed with 33 ml of NaH₂PO₄ and final volume was made to 1lit with DW.

50mM Working Phosphate Buffer, pH 7.4:
81 ml of Na₂HPO₄ was mixed with 19 ml of NaH₂PO₄ and final volume was made to 400 ml with DW.

1.4M NaCl:
8.18 gm of NaCl was dissolved in 20mM phosphate buffer, pH 7.1 to get a final volume of 100 ml.

2M GnHCl (Guanidinium hydrochloride):
19.106 gm of GnHCl was dissolved in 20mM phosphate buffer, pH 7.1 to get a final volume of 100 ml.

1.5M (NH₄)₂SO₄ (Ammonium sulphate):
99.1 gm of [(NH₄)₂SO₄] was dissolved in 20mM phosphate buffer, pH 7.1 to get a final volume of 500ml. The table below shows the volumes of various buffers mixed for preparing the elution grades.
1.5M (NH₄)₂SO₄ & 100 ml & 20mM phosphate buffer & \\
1M (NH₄)₂SO₄ & 66.7 ml & 33.3 ml & \\
0.75M (NH₄)₂SO₄ & 50 ml & 50 ml & \\
0.5M (NH₄)₂SO₄ & 33.3 ml & 66.7 ml & \\
0.25M (NH₄)₂SO₄ & 16.7 ml & 83.3 ml & \\
0.2M (NH₄)₂SO₄ & 13.3 ml & 86.7 ml & \\
0.15M (NH₄)₂SO₄ & 10 ml & 90 ml & \\
0.1M (NH₄)₂SO₄ & 6.7 ml & 93.3 ml & \\
0M (NH₄)₂SO₄ & — & 100 ml & \\

0.05M Tris-HCl, pH 7.4:

6.055 gm of Tris was dissolved in 800ml of DW. The pH of the solution was set to 7.4 with conc HCl and final volume made to 1000ml. The table below shows the volumes of various buffers mixed for preparing the elution grades.

<table>
<thead>
<tr>
<th>Amt of NaCl for 100ml solution</th>
<th>0.05M Tris-HCl, pH7.4</th>
<th>100 mM</th>
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<td>115 mM</td>
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<tr>
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<td>100 ml</td>
<td>0.731 gm</td>
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<tr>
<td>135 mM</td>
<td>100 ml</td>
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<tr>
<td>150 mM</td>
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<td>0.878 gm</td>
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</tr>
<tr>
<td>200 mM</td>
<td>100 ml</td>
<td>1.17 gm</td>
<td></td>
</tr>
</tbody>
</table>
Buffers for trypsin assay and chymotrypsin assay:

Trypsin Solution:

1 mg/ml of trypsin solution was made in 0.001 N HCl.

0.05 M Tris, pH 7.4, 0.02 M CaCl₂:

0.605 gm of Tris and 0.222 gm of CaCl₂ were dissolved in 80 ml of DW. Set pH to 7.4 with conc HCl and made up the volume to 100 ml with DW.

Soybean Trypsin Inhibitor:

1 mg/ml of trypsin inhibitor was made in 0.05 M Tris, pH 7.4, 0.02 M CaCl₂.

1 mM BAPNA Solution:

4.35 mg of BAPNA was dissolved in 100 μl of DMSO. Final volume as made to 10 ml with 0.05 M Tris, pH 7.4, 0.02 M CaCl₂.

Chymotrypsin Solution:

1 mg/ml of chymotrypsin solution was made in ice cold 0.001 M HCl.

0.067 M Potassium Phosphate Buffer, pH 7.0:

3.53 gm of KH₂PO₄ and 7.07 gm of K₂HPO₄ were dissolved in 800 ml of DW. Set pH to 7.0 and make final volume to 1 l with DW.

ATEE Solution:

2.35 mg of ATEE was dissolved in 10 ml of 0.067 M potassium phosphate buffer, pH 7.0. The solution was kept at 70°C to dissolve. It was cooled immediately and stored at room temp. The absorbance of the final solution to be used should be nearly 1.2 at 237 nm against the potassium phosphate buffer, pH 7.0 taken as blank.
Buffers for SDS-PAGE and Western Blot:

30% Acrylamide / 0.08% bisacrylamide:

29.2 gm of acrylamide and 0.8 gm of bis-acrylamide were dissolved in 80ml DW and final volume of solution was made to 100ml. Left the solution for a few hours overnight at room temp in a dark bottle for the solutes to mix properly in the solvent. Filtered through 0.45μm filter and stored at 4°C in dark.

1.5M Tris-HCl (4X)/SDS, pH 8.8:

18.2 gm of Trizma base was dissolved in 60ml of DW. Set pH to 8.8 with conc HCl. Added 0.4 gm of SDS to the solution and made up the volume to 100ml with DW.

0.5M Tris-HCl (4X)/SDS, pH 6.8:

3.025 gm of Trizma base was dissolved in 25 ml of DW. Set pH to 6.8 with conc HCl. Added 0.2 gm of SDS to the solution and made up the volume to 50 ml with DW.

10X Electrophoresis Buffer:

144 gm of glycine, 30.2 gm of Trizma base and 10 gm of SDS were dissolved in 800ml of DW. Final volume was made 1000 ml to prepare a stock solution of electrophoresis buffer (10X). The working electrophoresis buffer solution was 1X and was made at the time of use by diluting 100 ml of 10X stock solution to a final volume of 1000 ml.

0.02% Staining Solution:

0.2 gm of Coomassie Brilliant Blue R250 Dye (CBB) was dissolved in 500 ml of methanol. To this 50 ml of acetic acid was added. The final volume was made to 1000ml. The solution was filtered before use.

Destaining Solution:

50 ml of methanol (5%) was mixed with 70 ml of acetic acid (7%) and final volume was made to 1000 ml with DW.
Transfer Buffer for Western:

150 ml of 10X electrophoresis buffer, 300 ml of methanol, final volume to 1.5 lit with DW.

5X Solubilizing Buffer: 250mM Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue (BB), 20% glycerol, 500mM β-mercaptoethanol (β-ME):

1.51 gm of Trizma base was dissolved in DW and pH set to 6.8 with conc HCl. 0.25 gm of bromphenol blue and 5 gm of SDS was dissolved in Tris solution gradually adding small amounts of SDS. To this 25 ml of β-ME was added and final volume of the solution was made to 50ml with DW.

25 mM Tris-HCl pH 8.0/0.83% NaCl/0.02% KCl (TBS : Tris Buffered Saline)(10X):

15.13 gm of Trizma base, 41.5 gm of NaCl and 1 gm of KCl were dissolved in 400 ml of DW and set the pH to 8.0. Made the volume to 500 ml to get a final solution of 10X TBS. Working TBS solution (1X) was made at the time of use by diluting 100 ml of 10X stock solution to a final volume of 1000 ml.

0.05% TBST (0.05% Tween-20 in TBS):

50μl of Tween-20 was dissolved in 100 ml of 1X TBS to get the desired solution.

Buffer for preparation of the brain cytosolic proteins:

Homogenizing Buffer:

54.768 gm of sucrose and 0.727 gm of Tris were dissolved in 400ml of DW. Set pH to 7.4 with conc HCl. The final preparation gave the Sucrose-Tris homogenizing buffer containing 0.32M sucrose, 12mM Tris, pH 7.4.

Buffers for perfusion and fixation:

0.9% Phosphate Buffered Saline (PBS):

0.9 gm of NaCl was dissolved in 100 ml of 0.1M phosphate buffer, pH 7.4.
4% Paraformaldehyde:

4 gm of paraformaldehyde was dissolved in 100 ml of 0.1M phosphate buffer, pH 7.4.

30% Sucrose Solution:

30 gm of sucrose was dissolved in 100 ml of 0.1M phosphate buffer, pH 7.4.

Gelatin Adhesive:

1.5 gm of gelatin was dissolved in 7 ml of glacial acetic acid by heating slightly until clear. 90 ml of 30% alcohol was added to the gelatin solution. 0.1 gm of chrom alum was dissolved in 2 ml DW separately and added to gelatin solution to get the gelatin adhesive. The slides were placed in metal slide holder and allowed to sit in gelatin adhesive for 2-3 min. The slides were then dried overnight at 37°C.

Buffers for total RNA isolation and Northern Blot:

10X MOPS Electrophoresis Buffer:

41.8 gm of MOPS was dissolved in 700 ml of sterile DEPC treated Milli Q water. Set pH to 7.0 with 2N NaOH. 20 ml of DEPC treated 1M sodium acetate and 20 ml of DEPC treated 0.5M EDTA (pH 8.0) were then added. The volume of the solution was adjusted to 1 lit with DEPC treated Milli Q water. The final solution had the composition 0.2M MOPS (pH 7.0), 20mM sodium acetate and 10mM EDTA (pH 8.0). Sterilized the solution by passing it through a 0.45μm Millipore filter, and stored it at room temperature protected from light. (Note: The buffer yellows with age if it is exposed to light and is autoclaved.)

10X Formaldehyde gel loading Buffer:

50% glycerol, 10mM EDTA (pH 8.0), 0.25% bromophenol blue and 0.25% xylene cyanol.
1% Agarose gel containing 2.2M formaldehyde:

0.5 gm agarose was heated in 42 ml of DEPC treated Milli Q water. 3 ml of formaldehyde and 5 ml of 1X MOPS were added when the agarose cools down to 55°C.

20X SSC (Salt of Sodium Citrate):

175.3 gm of NaCl and 88.2 gm of sodium citrate were dissolved in 800 ml of DEPC treated Milli Q water. Set the pH to 7.0 with a few drops of a 14N solution of HCl. The volume was adjusted to 1 lit with DEPC treated Milli Q water. Dispensed into aliquots and sterilized by autoclaving. The final concentration of the ingredients was 3.0M NaCl and 0.3M sodium citrate.

Prehybridization/Hybridization Solution (for hybridization in phosphate-SDS buffer):

0.5M phosphate buffer (pH 7.2), 1mM EDTA, 7% (w/v) SDS, 1% (w/v) bovine serum albumin. An electrophoresis grade of bovine serum albumin was used.

0.5M EDTA, pH 8.0:

186.1 gm of disodium EDTA.2H2O was added to 800 ml of DW. The solution was stirred vigorously on a magnetic stirrer. The pH was set to 8.0 with NaOH (~20 gm of NaOH pellets). (Note: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to 8.0 by the addition of NaOH.)

Buffers for plasmid isolation:

0.8% Agarose in 1X TBE for DNA gels:

0.8 gm of agarose was dissolve in 100 ml of 1X TBE by heating

6X DNA gel loading dye:

The dye contains 0.25% BB, 0.25% xylene cyanol and 30% glycerol in water.
**5X Tris-Borate EDTA Buffer (TBE):**

54 gm of Trizma base and 27.5 gm of boric acid was dissolved in 300 ml of DW and 20 ml of 0.5M EDTA (pH 8.0) was added. Final volume was made to 500ml with DW to get a final solution of 10X TBE. Working TBE solution (1X) was made at the time of use by diluting 100 ml of 10X stock solution to a final volume of 1lit.

**PCI Solution (25:24:1):**

25 ml of equilibrated phenol, 24 ml of chloroform and 1 ml of isoamyl alcohol were mixed to get the PCI solution.

**RNase A Solution:**

The stock solution (10mg/ml) was diluted with Tris-HCl, pH 7.4 to get a working concentration was 50μg/ml of DNA solution.

**Solution I (Cell Suspension Solution):**

1ml of 0.5 M glucose and 250μl of 1M Tris-HCl, pH 8.0 were added in 5 ml of DW. 200μl of 0.5M EDTA, pH 8.0 was added and final volume made to 10 ml with DW.

**Solution II (Cell Lysis Solution):**

0.08 gm of NaOH was dissolved in 5 ml of DW. 0.1 gm of SDS was added and made final volume to 10 ml with DW to get 10 ml of Solution II.

**Solution III (Neutralization Solution):**

60 ml of 5M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of DW were mixed to get 100 ml of Solution III.
# List of Chemicals

The chemicals, solvents and biochemicals used along with the name of the supplier are as follows:

<table>
<thead>
<tr>
<th>Name of the chemical</th>
<th>Name of the supplier</th>
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