Presynaptic Membrane Receptor in Human Brain

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Abstract Myasthenia gravis (MG) is an autoimmune disease that results from antibody mediated damage of Acetylcholine receptor (AChR) at the neuromuscular junction. The autoimmune character of MG and pathogenic role of AChR antibodies have been established by several workers i.e., the demonstration of anti-AChR antibodies in about 90 % of MG patients. It has been demonstrated that patients with MG also have antibodies against a second protein named presynaptic membrane receptor (PsmR), which is identified by utilizing β -Bgtx, a ligand which binds to PsmR. Using β -Bgtx Sepharose 4B affinity matrix, the PsmR was purified from different regions of human cadaver brain by affinity chromatography. Purified receptor was characterized both by biochemical and immunological procedures. PsmR purified from different regions of the brain shows a specific activity of 0.37 ± 0.01 , 0.39 ± 0.02 and 0.43 ± 0.005 nM/ μ g of protein in Parietal lobe,

Occipital lobe and Frontal lobe respectively. The affinity purified PsmR from the brain of 87 and 68 kd (parietal lobe, occipital lobe and frontal lobe) shows immunoreactivity with myasthenic sera. These findings suggest that PsmR from brain is another antigen against which autoantibodies are developed in Myasthenia gravis patients. Upon treatment with various enzymes we concluded that PsmR from brain is a glycoprotein in which the immunoreactivity resides in the carbohydrate as well as the peptide epitopes. In conclusion the PsmR is another antigen against which autoantibodies are formed in different regions of brain. These can be used as a diagnostic tool for detecting antibodies in the sera or cerebrospinal fluid of MG patients.

Keywords Presynaptic membrane receptor · Human cadaver brain · β -Bgtx and purification

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Introduction

Myasthenia Gravis (MG) is an autoimmune disease that results from antibody-mediated damage of acetylcholine receptor (AChR) at the neuromuscular junction [26, 37, 22]. Much evidence has been presented which supports this hypothesis, including the induction of experimental autoimmune MG (EAMG) by immunization with AChR [26]. Anti-AChR antibodies acting on the AChR may thereby causes the abnormal muscular fatigue and other signs of MG [22, 19]. The autoimmune character of MG and pathogenic role of AChR antibodies have been established by several workers e.g.; the demonstration of anti-AChR antibodies in about 90 % of MG patients [21, 22, 2, 9], passive transfer of disease with IgG of MG patient to the mouse [35], localization of immune complexes (IgG and complement) on the postsynaptic membrane. Antibodies

against acetylcholine receptor (AChR) can be detected in most patients with Myasthenia gravis and are known to be involved in the immunopathogenesis of this disease. It has been demonstrated that patients with MG also have antibodies against a second protein named presynaptic membrane receptor (PsmR), which is identified by utilizing β -Bgtx, a ligand which binds to PsmR [23, 29]. PsmR represents another antigen besides AChR relevant for development of MG. In addition to postsynaptic nAChRs and presynaptic membrane receptor antibodies, titin and ryanodine receptor autoantibodies in MG patients showed correlation to severity of disease [1]. Some myasthenia gravis (MG) patients do not have detectable acetylcholine receptor antibodies and are termed as seronegative. The seronegative MG patients have antibodies to muscle specific tyrosine kinase (MuSK) [14]. These antibodies are directed against extracellular domain of MuSK and inhibit agrin induced AChR clustering in muscle myotubes [36] which specifically reacts with plasma from seronegative (70 %) and not from seropositive MG patients. Ryanodine receptor antibodies are often associated with malignant thymoma [33]. Apart from MG with thymoma, anti-titin antibodies have been observed to be an exclusive feature of late-onset MG [34].

Presynaptic receptors can be defined as receptors at or near the nerve terminal that can positively or negatively modulate transmitter release, that directly or indirectly influence the probability of an action potential resulting in exocytosis. It has been earlier shown that antibodies directed against β -Bgtx binding protein occur in MG [4, 25]. This protein named presynaptic membrane receptor (PsmR) has been isolated from human cadaver muscle [29], electroplax tissue of *Torpedo californica* [28], bovine diaphragm muscle [23] and fetal bovine diaphragm muscle [25] by utilizing β -Bgtx.

Materials and Methods

Human cadaver tissues was made available by AIIMS mortuary.

Clinical samples were collected from patients with MG from OPD or wards of Neurology department AIIMS. Control sera was obtained from healthy individuals. Beta bungarotoxin (β -Bgtx), Tween-20, orthophenylenediamine, bovine serum albumin (BSA) benzethonium chloride, benzamidinium hydrochloride, phenylmethyl sulphonyl fluoride, bacitracin, trypsin, sodiummetaperiodate, lipase, Sephadex G-25, CNBr activated Sepharose 4B were all purchased from Sigma Aldrich USA. Anti-human IgG-HRPO was purchased from Dako, Denmark Radio-isotope carrier free ^{125}I was purchased from Saxsons Biotech Ltd., India. Glass filter discs 2.5 cm were purchased from

Whatman Co., USA. Nitrocellulose sheets (0.45 μm) were purchased from MDI, India. All other reagents used were analytical grade (AR).

Membrane Preparation and Solubilization of Receptor

The receptors from different regions of brain tissues and liver tissues were solubilized according to the method as described by Jailkhani et al. [16, 17]. Briefly the tissues were minced and homogenized at 4 °C in 4 volumes of 0.01 M phosphate buffer pH 7.4 containing 0.1 M NaCl, 0.02 % NaN_3 , 0.001 M EDTA, 0.1 M benzethonium chloride, 0.002 M benzamidinium hydrochloride, 0.0001 M phenylmethyl sulphonyl fluoride (PMSF) and 0.5 mg/ml bacitracin (homogenizing buffer). The homogenate was then centrifuged at 20,000 $\times g$ for 60 min at 4 °C. The pellet obtained was suspended in 4 volumes of homogenizing buffer, aliquoted and were stored at -20 °C as membranes.

For the solubilization of membrane proteins (receptor) the pellet obtained at 20,000 $\times g$ centrifugation of tissue homogenate was extracted for 3 h at 4 °C in 2 volumes of homogenizing buffer containing 2 % (v/v) triton X-100. The supernatant (triton extract) obtained on centrifugation at 20,000 $\times g$ for 60 min was filtered through glass wool, aliquoted and were stored at -20 °C or below as a source of solubilized receptor.

Protein Estimation

The protein concentration of different preparation of antigens were determined by the method of Lowry using BSA as a standard [24]. However in case of triton extracts 2 % (v/v) of triton X-100 was used in the standard protein solution and samples centrifuged (in order to remove the precipitate formed) before reading the absorbance [11].

Radio-Iodination of Toxin (β -Bgtx)

Radio-iodination of toxin was done by iodogen (1,3,4,6-tetrachloro 3 α ,6 α -diphenylglycouril) method as described for iodination of α -Bgtx [16, 17]. Iodogen was solubilized in dichloromethane and added into glass tubes. A thin film was formed in the tubes by gentle swirling of nitrogen. To the precoated tubes, 10 μl of phosphate buffer (0.5 M pH 7.4), 2–5 μg of β -Bgtx in phosphate buffer and 0.5–1.0 mCi of (^{125}I) Na were added in sequence and the total volume was made to 100 μl . The reaction mixture was incubated for 5–15 min followed by addition of 20 μl of 2 % KI. The contents were gently mixed and filtered onto a column of Sephadex G-25 for separating radio-iodinated toxin from free iodine. Elution was done at room temperature with phosphate buffer (0.01 M, pH 7.4) containing 0.1 % BSA.

Binding Assay for Receptor Activity

The activity of PsmR in membrane preparation and triton extract of brain tissues was determined by virtue of its high affinity for β -Bgtx. Radio-iodinated β -Bgtx was used as a ligand. The free and bound ligand was separated by rapid filtration method (in case of membrane preparations) and ammonium sulphate precipitation method (in case of triton extracts i.e. solubilized receptor).

Rapid Filtration Method (for Membranes)

Briefly the membrane suspension was incubated with (^{125}I)- β -Bgtx in 0.01 M phosphate buffer, pH 7.4 containing 0.1 % BSA for 30 min at room temperature. Incubation was followed by addition of 5 ml of ice cold 0.01 M phosphate buffer, pH 7.4. The reaction mixture was then filtered on glass filter disc (2.5 cm). The disc was then washed 3 times with ice cold 0.01 M phosphate buffer (5 ml each) and counted for radioactivity in a γ -counter [15, 32].

Ammonium Sulphate Precipitation Method (for Solubilized Receptor)

(^{125}I)- β -Bgtx was incubated with triton extract for 60 min at 37 °C. 60 % saturated solution of ammonium sulphate was added and allowed to stand for 16 h at 4 °C. The precipitate was then filtered on GFC glass filter discs. The disc was washed 3 times with 30 % ammonium sulphate solution and the radioactivity was counted on a γ -counter [12]. To determine the non specific binding the samples were incubated in the presence of 100 fold excess of non radioactive toxin. The difference in total binding and non-specific binding counts represented the specific binding of the receptors.

Immunological Characterization of Presynaptic Receptor: ELISA

a. Indirect ELISA: The receptor was attached to the wells by coating the ELISA plates with the β -Bgtx in coating buffer (pH 9.6) and incubated for 16 h at 4 °C, followed by 5 washes with 0.1 M PBS (pH 7.4) containing 0.05 % Tween-20 (PBST). The triton extract diluted in PBST containing 0.1 % milk protein was added to wells for 2 h at 37 °C. After 5 washes, the suitably diluted test sera was added and allowed to react for 2 h at 37 °C. After 5 washes, anti-IgG-HRP conjugate was added and incubated for 2 h at 37 °C. After 5 washes, 0.2 ml of substrate O-phenylene diamine (40 mg/dl of 0.1 M citric acid, 0.2 M Na_2HPO_4 (pH 5.0) containing 40 μl of 30 % H_2O_2 was added

and allowed to react for 30 min at room temperature in dark. The reaction was stopped by 2.5 N H_2SO_4 . The OD was read at 492 nm in an ELISA reader [16, 17].

- b. Direct ELISA: in this method, the solubilized receptor was added directly to the wells of the micro-titer plate, without pre-coating the plate with β -Bgtx, rest of the steps can be used same as above.
- c. Competition ELISA: this was carried out to analyze the immunological cross reactivity of the presynaptic membrane receptors found in different tissues. Biologically active receptors was attached to the ELISA plate using β -Bgtx. The competing receptor was preincubated with the pooled myasthenic sera and left overnight at 4 °C before adding to the receptor pre-coated ELISA plate. Rest of the steps, were performed as earlier.

Purification of β -Bgtx Binding Protein

PsmR was solubilized from respective different regions of brain tissues using homogenizing buffer containing 2 % (v/v) triton X-100. The receptor molecule was purified by affinity chromatography on a β -Bgtx affinity gel column followed by elution with 1 M ammonium hydroxide. The isolated product was then run on a SDS-PAGE to find the molecular weight and subunit composition.

Coupling of β -Bgtx CNBr (Cynogen bromide) Activated Sepharose-4B

CNBr (Cynogen bromide) activated Sepharose-4B was used for affinity chromatography. CNBr activated Sepharose 4B was washed and swelled for 30 min in ice-cold HCl, followed by washing with distilled water. The gel was then washed with coupling buffer-0.1 M NaHCO_3 containing 0.5 M NaCl, with a pH of 8.3–8.5. The washed gel was then mixed with the protein solution (β -Bgtx dissolved in coupling buffer) and mixed overnight at 4 °C with gentle stirring. The supernatant was removed and the unreacted ligand washed away. The un-reacted groups on the gel were blocked by using 0.2 M glycine pH 8.0 for 16 h at 4 °C. Blocking was followed by 5 alternate cycles of washing with high and low buffer solutions: coupling buffer, pH 8.3–8.5 and 0.1 M acetate buffer, pH 4. The gel was then equilibrated with the buffer (homogenizing buffer with 0.5 M NaCl and 0.1 % v/v triton X-100).

Affinity Purification of β -Bgtx Binding Protein on β -Bgtx-Sepharose 4B Gel

The affinity gel was packed in a column and equilibrated with homogenizing buffer. The triton extract was loaded

and re-passed onto the affinity gel till nearly all the β -Bgtx binding protein was bound to gel. Washing was done extensively with homogenizing buffer containing 0.5 M NaCl and triton X-100 whose concentration was reduced from 1 to 0.1 % v/v. The elution was done with 1 M ammonium hydroxide. The eluate was dialyzed against the homogenizing buffer to remove the ammonium hydroxide. The concentrated protein was then assessed for immunological characteristics (ELISA with myasthenic pool sera), toxin binding (radio-iodinated β -Bgtx), nature and molecular weight (SDS-PAGE).

Characterization

SDS-PAGE: For knowing the subunit composition each antigen preparation was mixed with an equal volume of 2 $\times$ sample buffer (containing 0.5 M Tris HCl pH 6.8, 20 % SDS, 20 % glycerol, 0.1 M EDTA, 25 % β -mercaptoethanol and 0.1 % bromophenol blue) and heated at 100 °C for 10 min. The supernatant was loaded on the gel. SDS-PAGE was conducted in 10 % polyacrylamide resolving gel and 5 % stacking gel containing 1 % SDS [18].

Electrophoresis was performed at a constant current of 30 mA until bromophenol blue reached the bottom of the gel.

Transfer

The protein from SDS-PAGE gel was transferred onto nitrocellulose sheet after equilibration in tris glycine buffer. Transfer was carried out in a wet type transfer system at 15 V for 16–18 h at 4 °C.

The nitrocellulose membrane after transfer of proteins was blocked by 5 % milk powder in Tris buffer (containing 50 mM Tris pH 7.5, 150 mM NaCl with 0.05 % Tween-20 TTBS). The nitrocellulose paper was immersed in pooled myasthenic sera for 1 h at room temperature. The membrane was washed thrice with TTBS and then incubated for 1 h at room temperature in anti-human IgG-HRP conjugate diluted in tris buffer saline with 5 % milk powder. The membrane was washed with TBS and the incubated with substrate solution (5 mg Diaminobenzidine in Tris buffer saline with 5 μ l H₂O₂) and reaction was stopped by washing the blot once with TBS.

Treatment with Sodium Metaperiodate and Enzymes

In order to identify the nature of purified receptor we followed the previous published methodology [29]. The purified proteins from parietal lobe of human cadaver brain was coated directly at pH 9.7 on ELISA plates and treated with sodium metaperiodate and the enzymes, trypsin, lipase and glucosidase, for 3 h at 37 °C. After washing the

ELISA was carried out as described earlier and the effect on immunoreactivity with the myasthenic pool sera tested.

Results

Radio-Iodinated Profile of β -Bgtx

β -Bgtx were iodinated by the iodogen method as described in the methodology [15]. The elution profile is shown in Fig. 1. The peak fractions (radio-iodinated toxins) were pooled and counted in a gamma counter and their specific activity was calculated. Specific activities of three preparations of radio-iodinated β -Bgtx are given in Table 1.

Binding of radio-iodinated toxin (β) to membrane and triton extract of human cadaver brain regions was done by rapid filtration assay and ammonium sulphate precipitation method as described in the methodology. In addition to different regions of brain, specific binding of β -Bgtx was also studied in membranes and triton extract of liver. Specific binding was expressed in terms of fmol/mg tissue as shown

Fig. 1 Elution profile of radio iodinated β Bgtx. To the precoated iodogen tube, phosphate buffer, β Bgtx and (125 I) Na of 0.5 mCi were added in sequence and the resultant volume was made to 100 μ l. The mixture was incubated for 15 min at RT followed by addition of 20 μ l of 2 % KI. The contents were gently mixed and transferred onto Sephadex G-25 column for separating toxin from free iodine. Elution was done at RT with 0.01 M phosphate buffer containing 0.1 % BSA. 1 ml of each fraction was collected and counted in a gamma counter. The radio-iodinated peak fractions were pooled and specific activity was calculated as shown in Table 1. The experiment was done three times

Table 1 Specific activities for radio-iodinated β Bgtx prepared by iodogen method

	Toxin (μ g/ μ l)	125 I (mCi)	Pool volume (ml)	Specific activity (cpm/nmol)
1.	2/10	0.5	2.8	3.8×10^8
2.	2/10	0.5	4	8×10^8
3.	2/10	0.5	3	3×10^8

Table 2 Binding of radio-iodinated beta-Bgtx with membranes of cadaver tissues

Tissue	Counts per minute				
	Total	Non-specific ^a	Specific	mg/tissue	fm/mg tissue
Frontal lobe	69,280	13,594	55,686	4,454	5.5 ± 0.07
Occipital lobe	68,178	12,928	55,250	4,420	5.5 ± 0.07
Parietal lobe	65,990	11,978	54,012	4,321	5.4 ± 0.06
Temporal lobe	67,726	12,648	55,078	4,406	5.5 ± 0.07
Hypothalamus	66,888	12,382	54,506	4,360	5.4 ± 0.04
Hippocampus	67,790	10,494	57,306	4,584	5.7 ± 0.06
Cerebral Cortex	67,198	11,726	55,472	4,437	5.5 ± 0.07
Cerebellum	67,608	11,084	56,524	4,521	5.6 ± 0.06
Cerebrum	66,848	10,840	56,008	4,480	5.6 ± 0.06
Liver	81,407	42,507	0	0	0

^a Non specific CPM obtained in the presence of 100 molar excess of non radioactive toxin

Table 3 Binding of radio-iodinated beta-Bgtx with triton extract of human cadaver tissues

Tissue	Counts per minute				
	Total	Non-specific ^a	Specific	mg/tissue	fm/mg tissue
Frontal lobe	58,434	8,856	49,578	1,983	4.9 ± 0.06
Occipital lobe	57,476	9,346	48,130	1,925	4.8 ± 0.06
Parietal lobe	57,726	9,840	47,456	1,890	4.7 ± 0.06
Temporal lobe	57,726	8,648	49,078	1,963	4.9 ± 0.07
Hypothalamus	57,790	8,494	49,472	1,938	4.9 ± 0.07
Hippocampus	57,198	8,726	48,472	1,938	4.8 ± 0.06
C. Cortex	57,642	9,430	49,212	1,968	4.9 ± 0.06
Cerebellum	57,608	8,084	49,524	1,980	4.9 ± 0.07
Cerebrum	57,514	8,183	49,331	1,973	4.9 ± 0.06
Liver	60,412	60,514	0	0	0

in Tables (2, 3). Radio-labelled β -Bgtx binding sites was also observed in the membranes and triton extract in all the different regions of brain. No specific binding was observed in liver membranes and triton extract thereof indicating that liver does not contain any presynaptic receptor.

Immunoreactivity Profile of Different Cadaver Tissues with Myasthenic Sera

Fresh skeletal muscle was obtained from amputation case and was used as a source of receptor (PsmR). Using indirect ELISA [16, 17], the receptor in detergent solubilize extract (triton extract) were added on ELISA plates pre-coated with β -Bgtx. It was then incubated with pooled myasthenic sera. As evident from Fig. 2, the triton extract gave a concentration dependent immunoreactivity with MG pooled sera, while no immunoreactivity was seen with control pool sera. Since the present research aims to achieve purification of PsmR (β -Bgtx binding protein), large amount of tissue was required. Fresh human tissues is neither considered to be ethical nor it is feasible to obtain, therefore, the use of cadaver tissues was preferred which were obtained from the mortuary of AIIMS. It is evident

from Fig. 3 that cadaver skeletal muscle gave comparable immunoreactivity with MG pool sera same as given by the fresh skeletal muscle, thereby justifying its use as a source for the PsmR. In triton extract of different regions of brain, the immunoreactivity profile of PsmR with myasthenic sera was low as seen in Fig. 3. Liver triton extracts showed no immunoreactivity with myasthenic sera [29], which indicated that liver can be used as negative control.

Purification of PsmR (β -Bgtx binding protein) from Different Regions of Human Cadaver Brain

Purification of PsmR was done by affinity chromatography, using β -Bgtx Sepharose 4B matrix

1. Coupling of β -bungarotoxin to CNBr activated sepharose 4B

For PsmR, β -Bgtx was found to be a specific ligand. β -Bgtx was coupled to CNBr-activated Sepharose 4B at a concentration of 3 mg/g of the dry gel. The actual amount of β -Bgtx coupled with the gel was determined by competition ELISA (Fig. 4). The precoupling toxin solution and post coupling

Fig. 2 Immunoreactivity of triton extracts from fresh skeletal muscle and human cadaver muscle. Indicated dilutions of triton extracts from fresh and cadaver skeletal muscle were reacted with β -Bgtx pre coated plates for 2 h at 37 °C, to trap the presynaptic membrane receptor (β -Bgtx binding proteins) receptors, which were then sequentially reacted with pooled myasthenic sera/control sera (1:200), anti IgG-HRP conjugate and substrate (OPD), the OD obtained at 492 nm at each input triton extracts dilutions are shown in figure

supernatant were assessed for their ability to compete with known concentration of β -bungarotoxin for binding PsmR in the triton extract. In the three preparations, 43.7, 42 and 46.6 % of the toxin were found to be coupled with the gel. The degree of coupling was also determined by taking OD at 280 of the pre and post coupling toxin solution. The OD at 280 nm decreased from 0.46 to 0.061 after the coupling procedure. The affinity gel was blocked and equilibrated with homogenizing buffer containing protease inhibitors and poured into a disposable column at 4 °C prior to use.

2. Affinity purification of PsmR

For purification of PsmR we followed the procedure [29]. The immunoreactivity and toxin binding profile of the fractions eluted from the affinity column for parietal lobe of the brain is shown in Fig. 5. The details of affinity purification of PsmR from different regions of the brain are shown in Table 4. PsmR was affinity purified from 3 sets of triton extract obtained from different regions of the brain. The mean specific activity, recovery and fold purification are shown in Table 4.

SDS-PAGE and Western Blot

The SDS-PAGE profile of triton extract of different regions of the brain are shown in Fig. 6a. Triton extracts of different brain regions contain more proteins as compared to muscle. Figure 6b shows SDS-PAGE of purified preparation from the parietal lobe preparations showed 6–7 prominent bands corresponding between 105 and 48 kd (Fig. 6b). The reactivity of the purified PsmR (β -Bgtx binding protein) from different regions of the brain was also determined by immunoblotting. In the purified protein from different regions of the brain, two bands were observed of 87 and 68 kd (Fig. 6c). No reactivity was seen with control pooled sera (Fig. 6d). This indicated that only 2 subunits of purified receptors from different regions of the brain are immunoreactive.

Presynaptic Membrane Receptor Is a Glycoprotein

The effect of various enzymes on immunoreactivity of purified PsmR of the parietal lobe of brain are shown in Fig. 7. The purified PsmR were subjected to enzymatic treatment with trypsin, glycosidase, lipase, sodium meta periodate and the effect on the immunoreactivity with pooled MG sera was assessed by direct ELISA. Treatment with sodium metaperiodate, glucosidase and trypsin shows a significant decrease in the immunoreactivity of purified PsmR of parietal lobe of brain, whereas lipase did not produce any effect. These result suggest that PsmR (β -Bgtx binding protein) of brain is a glycoprotein as immunoreactivity resides in the carbohydrate as well as the peptide epitopes which is similar to as purified PsmR from human cadaver muscle [29].

Immunological Characteristics

The affinity purified PsmR (β -Bgtx binding protein) from different regions of the brain showed immunological cross reactivity with each other while liver triton extract did not show any competition (Fig. 8).

The specific binding of 125 I- β Bgtx binding in the purified PsmR preparations and the corresponding triton extracts from different regions of the brain was demonstrated by competition with its cold β -Bgtx as shown in Figs. 9 and 10. The binding characteristics Bmax and kD calculated from the Scatchard plots (Table 5). The binding characteristics of the PsmR from different regions of the brain are nearly identical.

Discussion

It was previously observed that in MG a decrease in the number of acetylcholine receptors at the neuromuscular

Fig. 3 Immunoreactivity of triton extracts from different regions of brain of different cadavers. Indicated dilutions of triton extracts from different regions of human brain were reacted with α -Bgtx and β -Bgtx pre coated plates for 2 h at 37 °C, to trap the presynaptic membrane receptor (β -Bgtx binding proteins) receptors, which were then sequentially reacted with pooled myasthenic sera/control sera (1:200), anti IgG-HRP conjugate and substrate (OPD) the OD obtained at 492 nm at each input triton extracts dilutions are shown in figure

Fig. 4 Coupling of β -bungarotoxin to CNBr activated Sepharose 4B. Competition ELISA was used to determine the amount of β -bungarotoxin left in the supernatant after overnight coupling with CNBr activated Sepharose 4B. Different concentrations of β -bungarotoxin were coupled with the antigen (triton extract of muscle) overnight at 4 °C before addition to the β -bungarotoxin (1 μ g/ml) wells. A standard curve was prepared and the unknown concentration in the post-coupling supernatant calculated

Fig. 5 Affinity purification on β -bungarotoxin Sepharose 4B column. 40 ml of Triton extract from parietal lobe of cadaver brain were passed (3 times) through β -Bgtx Sepharose 4B column. After extensive washing (100 ml), the receptor was eluted with 1 M NH_4OH . 1 M fractions were collected, dialyzed to remove NH_4OH and then assessed by indirect ELISA (as indicated in purple line) and radioiodinated β -Bgtx binding (as indicated in filled circles)

junctions causes fatigability of the muscle [8]. This was first identified by use of radiolabelled snake toxin α -Bgtx, which binds specifically, quantitatively and irreversibly to

acetylcholine receptor of skeletal muscles [3]. α -Bgtx, has been long used for specific identification, quantification and purification of the receptor [27, 6]. The AChR binds to

Table 4 Affinity purification of presynaptic membrane receptor (PsmR) from different regions of brain

Purified protein								
T. ext (ml)	Binding (nmol)	Protein(mg)	Sp. activity (nmol/ug)	Binding (nmol)	Protein (mg)	Sp. activity (nmol/μg)	Recovery (%)	Fold purification
Brain (parietal lobe)								
40	16.2	89	0.0001820	6.4	0.017	0.37	40	2,032
40	16.1	86	0.0001872	6.3	0.017	0.37	39	1,976
40	15.8	89	0.0001775	6.8	0.018	0.37	43	2,084
Occipital lobe								
40	16.8	87	0.00019310	6.7	0.017	0.39	39	2,019
40	16.4	85	0.00019294	6.8	0.016	0.42	41	2,182
40	16.6	84	0.00019761	6.5	0.017	0.38	39	1,922
Frontal lobe								
40	16.7	86	0.00019418	6.9	0.016	0.43	41	2,214
40	16.6	85.4	0.00019437	6.7	0.015	0.44	40	2,263
40	16.4	86	0.00019069	6.9	0.016	0.43	42	225
			Parietal lobe		Occipital lobe		Frontal lobe	
Specific activity			0.37 ± 0.01		0.39 ± 0.02		0.43 ± 0.005	
Recovery			33.5 ± 2.08		39.6 ± 1.15		41 ± 1	
Fold purification			2030 ± 54		2041 ± 131.3		2243 ± 26

Fig. 6 SDS-PAGE and Western blot of different regions of cadaver brain. Triton extract from different regions of brain were run on SDS-PAGE using a stacking gel of 5 % and a resolving gel of 10 %. Electrophoresis was carried out at 100 V for 3 h. The gel was stained with Commassie brilliant blue as shown in Fig. 6a. Affinity purified β -Bgtx binding presynaptic receptor from different regions of brain

α -Bgtx, a toxin that is used to isolate AChR from crude receptor preparation [10]. The receptor in its monomeric form has a sedimentation coefficient of 9S and has a

(5 μ g protein) were run on SDS-PAGE using a stacking gel of 5 % and a resolving gel of 10 %. Electrophoresis was carried out at 100 V for 3 h. The gel was containing the purified protein was silver stained as shown in Fig. 6b. Affinity purified presynaptic receptor from different regions of brain were probed with Myasthenic sera pool and control sera at a dilution of 1: 500 as shown in Fig. 6c, d respectively

subunit composition of 40, 50, 60 and 65 kd [6]. Experimental autoimmune MG (EAMG) is also induced by passive transfer of IgG or sera from patients with MG to mice,

Fig. 7 Effect of periodate and enzymes on immunoreactivity of β -Bgtx binding receptor. Purified receptor from Parietal lobe was coated onto ELISA plate wells by direct coating at pH 9.6 for 2 h at 37 °C. 200 μ l of sodium metaperiodate (50 mg/ml), trypsin (1 mg/ml), lipase (0.5 mg/ml) and glucosidase (0.25 mg/ml) were then added to the wells and incubated for 3 h at 37 °C. Untreated. After washing, the receptor was sequentially reacted with MG sera pool and HRPO conjugated secondary antibody as usual and the effect on immunoreactivity assessed

Fig. 8 Competition ELISA of affinity purified receptor from parietal lobe of cadaver brain. 100 fmol of affinity purified β -Bgtx receptor was coated onto ELISA plate through 1 μ g/ml β -Bgtx. Indicated concentrations of the competing antigens were pre-incubated with the MG sera pool (1:200 dilution at overnight at 4 °C) and then reacted with the coated antigen followed by the conjugate and substrate

or from rats with EAMG to healthy rats [20]. Anti-AChR antibodies have been detected in serum from 63 to 93 % of MG patients [13, 38]. It has been considered that a significant role of nAChRs in CNS may be to modulate as well as to mediate transmission [7]. Presynaptic nAChRs modulate (H^3) adrenaline release and constitutes $\alpha 3$ and $\beta 4$ subunit composition [5].

It has been demonstrated that Patients with MG have also antibodies against a second protein which is called

presynaptic membrane receptor (PsmR), which has been isolated from human cadaver muscle and bovine diaphragm muscle utilizing β -Bgtx [29, 23, 40]. Antibodies of PsmR and AChR from MG patients sera showed about 45–55 % cross reactivity and there is high correlation between serum levels of both antibodies [40]. Cells secreting anti-PsmR antibodies belonging to the IgG, IgA isotypes and less frequently of the IgM isotype were detected in most MG patients [23]. Antibody secreting cells are more frequently found in bone marrow of seropositive myasthenic patients compared to seronegative patients [25]. There was a positive correlation between the numbers of PsmR-reactive and AChR-reactive T cells. In conclusion, the results show that PsmR-stimulated T cells secreted IFN-gamma and/or IL-4. This T cell response is MHC class II restricted. Thus, this study indicates that both Th1/Th2 or Th0 subsets of the T cells are involved in the autoimmune response in MG [41]. It has been observed that seronegative MG patients show antibodies against presynaptic membrane receptor [30]. The seronegative MG is an autoimmune disease and antibody secreting cells (ASC) to AChR and to prsmR are present both in the blood and the bone marrow in both seronegative and seropositive patients. A major difference between the groups lies in the significantly greater number of ASC found in the bone marrow in the seropositive cohort [25]. Striational antibody levels are elevated in seropositive MG patients, and they are rarely found in seronegative MG patients. Thus assays for this antibody is of limited value in confirming the diagnosis of MG. The main clinical value of striational antibodies is that their presence in the serum of an MG patient is associated with thymoma in a high percentage of cases. In particular, 60 % of patients with MG with onset before age 50 years who also have elevated striational antibodies also have thymoma [31].

The aim of present study was to purify presynaptic receptor (β -Bgtx binding protein) from different regions of human cadaver brain. The use of β -Bgtx CNBr activated Sepharose 4B affinity matrix was used for purification of Presynaptic receptor. The elution of the purified PsmR (β -Bgtx binding protein) was done with 1 M NH_4OH . The immune-reactivity and toxin binding profile of the fractions eluted from the affinity column for different regions of brain (Fig. 5) Earlier β -Bgtx binding protein was purified from human cadaver muscle, bovine diaphragm, torpedo by affinity chromatography using CNBr activated Sepharose 4B. In case of human skeletal muscle the bound protein was eluted with 1 M NH_4OH [29]. In bovine diaphragm and torpedo the bound protein was eluted with 0.5 M KCl, sequentially loaded on wheat germ lectin column and eluted by *N*-acetylglucosamine [40, 28]. The SDS-PAGE profile of purified PsmR (β -Bgtx binding protein) of different brain regions as shown in Fig. 6 showed two

Fig. 9 Effect of cold β -Bgtx on binding of radio-iodinated β -bungarotoxin. 50 μ l of triton extract from different regions of brain were incubated with 1.5×10^5 cpm of radio-iodinated β -Bgtx and competed with different concentration of non radio-active β -Bgtx.

The Scatchard plots were made by plotting (B)/(F) against (B). (B): concentration of toxin bound at each input concentration of cold toxin. (F): input concentration of toxin

Fig. 10 Effect of cold β -Bgtx on binding of radio-iodinated β -Bgtx to purified presynaptic receptor of different brain regions. 50 μ l of purified presynaptic receptor from different regions of brain were incubated with 1.5×10^5 cpm of radio-iodinated β -Bgtx and competed with

different concentration of non radio-active β -Bgtx. The Scatchard plots were made by plotting (B)/(F) against (B). (B): concentration of toxin bound at each input concentration of cold toxin. (F): input concentration of toxin

Table 5 Affinity and number of Beta-Bgtx binding sites in whole triton extract and purified PsmR's from different regions of brain

Tissue	Triton extract		Purified presynaptic receptor	
	Kd (nmol)	No. of binding sites (fmol/mg tissue)	Kd (nmol)	No. of binding sites (fmol/mg tissue)
Parietal lobe	1.37 ± 0.18	26.4 ± 2	2.15 ± 0.14	24.5 ± 2.1
Occipital lobe	1.33 ± 0.13	25.6 ± 2.6	2.25 ± 0.12	25.1 ± 2.1
Frontal lobe	1.5 ± 0.12	28 ± 1.8	2.15 ± 0.13	24 ± 2.2

prominent bands corresponding to 87 and 68 kd. Immunoreactivity profiles of purified PsmR (β -Bgtx Binding protein) from different brain regions with myasthenic sera was negatively affected by treatment with sodium-metaperiodate, glucosidase and trypsin, whereas no effect was seen on treatment with lipase. This provides evidence that purified PsmR (β -Bgtx binding protein) is a glycoprotein as reported earlier [29]. It has been previously observed that presynaptic proteins like synaptophysin are integral transmembrane glycoprotein [39]. The specificity of ^{125}I - β Bgtx binding in the purified presynaptic receptor preparations and the corresponding triton extracts of different regions of brain was demonstrated by competition with cold β -Bgtx and Scatchard plots were constructed. The binding characteristics Bmax and kd calculated from the Scatchard plots (Table 5). It was observed that binding of PsmR in triton extract and the purified PsmR is almost identical. Immunoblotting of the purified protein from different brain regions with pooled myasthenic sera gave a prominent bands of 87 and 68 kd (Fig. 6c) and no reactivity was seen with control sera pool. In conclusion the PsmR is another antigen against which autoantibodies are formed in different regions of brain. These can be used as a diagnostic tool for detecting antibodies in the sera or cerebrospinal fluid of MG patients.

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