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Biochemical characterization of neurolectins, neuron, glial cells, synaptosomes and cell subfractions of the brain

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A B S T R A C T

In the present work the results of experimental research in the field of quantitative and qualitative distribution of neurolectins in the neurons, glial cells, synaptosomes and subfraction of the rats, chickens and bovine brain are presented. Neurolectins were isolated from the enriched fraction of neural and glial cells The specific activity of neurolectins was 780 (neuron) and 713 (glia) units and showed diverse affinity for carbohydrates: neurolectin isolated from enriched fraction of neurons showed specific sensitivity to D-galactose (NL-Gal, 16.2 mM), neurolectin isolated from the enriched fractions of glial cells showed specific sensitivity to inositol (GL-I, 4.6 mm), and other carbohydrate haptens depending on the concentration. Two neurolectins were isolated from synaptic vesicles of chicken and bovine brain and were identified as specific to inositol (SVL-I) and N-acetyl-D-galactosamine (SVL-NAGA), which, according to our hypothesis, were actively involved in the capture and secretion of neurotransmitters and biological active substances. Since the quantitative distribution of neurolectins in the animal brain was established, it was of great interest to study the distribution of neurolectins at the level of subcellular fractions of nerve cells. It has been found that agglutination with mitochondrial neurolectin is specifically inhibited by glucose, galactose, and N-acetylglucosamine, N-acetyl-galactosamine and is called BML-GluG. Neurolectins of the nucleus nerve cells were separated on a Protein PAK-300-SW column. Nuclear neurolectins are divided into two protein fractions. Using the hapten-inhibitory method, it was shown that both neurolectins of the nucleus of the rat haptens of the brain are N-acetyl-D-glucosamine, D-galactose and L-fructose (NucL-G).

Keywords: Neurolectins, Brain, Neurons, Glial cells, Synaptic vesicles, Mitochondria.

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Introduction

Lectinology as a new area recently has been intensively developed. Lectins selectively and reversibly bind to carbohydrates and agglutinate cells and tissues. They actively participate in such vital processes as communication and signaling systems, adhesion, structural organization of brain, on transport and migration of nerve cells at the postnatal development and in brain termoregulation process etc.

Lectins are proteins with a low molecular weight and have glycoprotein nature. Based on the functions of lectins, great prospects arise for their use in medicine and biology. The anticancer, antiallergic and immunomodulating properties of lectins have already found practical application in clinical medicine for the diagnosis and treatment of cancer and infectious diseases.

Material and methods

Various compositions of extracting solutions for the extraction of neurolectins from the brain of embryonic and adult chickens, rats and bovine, were tested, among which the most promising were 0.5 mM Triton X-100 and 5 mM EDTA in potassium phosphate buffer (PPB). For further purification of lectins, a saturated solution of ammonium sulfate from 0 to 80% was used. Ratio of nerve cells to extracting solution was 1: 5 (mg/ml). The mixture was centrifuged at 16000 g for 15 minutes. For the gradual fractionation of ammonium sulphate at every step of sedimentation solution was added to the extract of soluble proteins. Excess of inorganic ions was removed by the dialysis on the G-10 Sephadex gel-filtration column (50x2.7 cm). The sediment

was dissolved in the minimum volume of PPB and its hemagglutination activity was determined.

Lectin activity was visually determined using microtitration method by Takachi [1] on immunologic slides using the hemagglutination test on trypsin-treated erythrocytes of rabbit. Lectin activity was evaluated by the minimum concentration of protein (mg/ml) which caused an agglutination of trypsin-treated rabbit erythrocytes. Lectin activity was determined in the following way: $SA=T^{-1}\cdot C^{-1}$, where T^{-1} (titre) is the degree of protein dilution in the last well of the titration plate, where hemagglutination still occurs, C- protein concentration in mg/ml.

Specificity of lectins to carbohydrates was studied using hapten-inhibitory method [2]. 0.6 M solutions of oligosaccharides on the basis of PPB were used for analysis. In the experiments the carbohydrates were used as haptens: D-galactose, methyl-D-galactose, N-acetyl-D-galactosamine, D-glucose, L-fucose, D-fructose, L-inositol, D-lactose, D-maltose. Hapten-specificity was judged by the minimum concentration of a carbohydrate (mM), which caused an inhibition of hemaggutinating activity of a lectin. For the agglutination reaction 2% suspension of trypsin-treated rabbit erythrocytes prepared on the PPB solution was used. Protein concentration was determined by Lowry method [3].

Results and discussion

Protein with agglutination ability was discovered in 1888 by Peter Hermann Stilmark in the seeds and oils of the Ricinus communis plant and was named Ricin (hemagglutinin). Since the detection of proteins with agglutinating ability is assigned to lectins [4], we called lectins excreted from nerve tissues neurolectins [5- 8].

The first neurolectin was isolated from the Electric Organ [9]. Lectin agglutinates rabbit erythrocytes treated with trypsin and is called electrolectin. Electrolectin was partially bound to the membrane, but it was also found in the soluble fractions of homogenates of electrical organs. Neurolectin was purified by means of affinity chromatography and isolated as a separate fraction with a molecular weight of 33,000 kDa. Large amounts of electrolectin were found in the microsomal fraction of the electric organ obtained after the centrifugation (70 min 100000 g) and it was increased to 11% of the total amount of proteins.

In 1987, Janeta and his colleagues isolated two endogenous soluble neurolectins CSL1 (Mr = 33,000) and CSL2 (Mr = 31,500) from a cerebellum of the brain of young rats with a 0.5% solution of Triton X-100. Using the immunoaffinity method, another minor component with a molecular mass of Mr = 45,000 was isolated from the same tissue, the minor components of which were CSL1 (mainly lysosomal) and CSL2- (mainly cytoplasmic) subunits. The aforementioned neurolectins turned out to be mannose-specific and were effectively extracted with a 0.5 M mannose concentration [10-13].

The discovery of neurolectins at different stages of the embryonic development of chickens has become a subject of our interest. First, in order to reveal the maximum activity of neurolectins in the brain of embryonic and adult chickens, various compositions of extracting solutions were tested, among which the most promising were 0.5 mM EDTA and potassium phosphate buffer (PPB). For further purification of lectins, a saturated solution of ammonium sulfate from 0 to 80% was used. Neurolectin activity under the conditions of embryonic development of chickens in the brain is found to be deprived on the 5th day and it reaches its maximum before hatching [14].

Noteworthy results were obtained by studying the distribution of neurolectins in the rat brain fractions P1 and P2 within 2-16 days before hatching [14]. Fractions P1 and P2 were obtained by the method of Teichberg and others [9]. P fractions were dissolved in 0.5 mM EDTA and then homogenized. After centrifugation, the precipitate was dissolved in PPB with sodium chloride and the lectin activity of the fractions P1 and P2 was studied [6, 7]. Significantly higher activity was manifested on the 7th day of embryonic development in the P2 protein fraction. If the specific activity of neurolectins before hatching of the P1 and P2 fractions was 79.37 and 132.50 units, respectively, then, after hatching, this indicator decreased to the maximum and increased to no more than 16.04 and 80.62 units [14]. It is assumed that a decrease in the activity of neurolectins is explained by an increase in brain mass and brain volume. A similar opinion was expressed by other authors [15].

The content of neurolectins was also studied in the brain of chickens under conditions of embryonic and postembryonic development [14, 16-18]. Neurolectins were extracted from the chicken brain with a solution of 5 mM EDTA PPB. Subsequently, the proteins were purified with ammonium sulfate at a saturation of 80-90%. The isolated neurolectin was specific for D-galactose (30 mM), D-lactose

(15 mM), N-acetyl-D-glucosamine and, therefore, it was designated as LC-GLN. Neurolectin was purified by lactose-sepharose column 4B affinity chromatography. As a result, lectin activity increased by more than 25 times. Using the method of electrophoresis in polyacrylamide gel, the molecular weight of neurolectin was 15 kDa. Lectin is a glycoprotein with a monosaccharide content of approximately 20%. Neurolectin showing an optimal activity at pH of 7.0-8.0, was particularly sensitive to Ca^{+2} ions, in the presence of a Ca^{+2} EGTA chelator, at a concentration of 0.2 mM, an agglutination of trypsinized red blood cells was completely inhibited [14, 18-20].

In 1982, mannose-specific neurolectin was isolated from the brain of embryonic and adult chickens, which was purified 1700 times using APL Sepharosa 4B. Such a polyacrylamide gel neurolectin preparation in the presence of sodium dodecyl sulfate was divided into two protein fractions with a molecular weight of 15 kDa and 13 kDa of lectin activity. Neurolectins extracted from different tissues of embryonic and adult chickens were identical. It should be noted that neurolectins from chicken muscles, the brain, and liver had similar chemical and immunological properties [15, 16].

Neurolectins from neurons, glial cells and synaptic vesicles

After the discovery of neurolectins in the brain of chickens and rats, we examined the quantitative distribution of neurolectins in enriched fractions of neurons, glial cells and in synaptic vesicles. Enriched fractions of neurons and glial cells were obtained by the modified Rose method [21, 22].

Neurolectins were isolated from the enriched fraction of neural and glial cells with a 0.5% solution of Triton X-100. After homogenization, the mixture was centrifuged (10000 g/30 min), the supernatant was fractionated with ammonium sulfate in different saturations. The residue was dissolved in an agglutinating PPB solution, and after dialysis, the activity of neurolectin was determined in each individual fraction. The specific activity of neurolectins was 780 (neuron) and 713 (glia) units, respectively. Neurolectins isolated from enriched fractions of neurons and glial cells showed a diverse affinity for carbohydrates. Namely, the neurolectin isolated from the enriched fraction of neurons, showed a specific sensitivity to D-galactose (16.2 mM) and N-acetyl-D-glucosamine (8.1 mm), neurolectin isolated from enriched fractions of glial cells showed specificity to D-fructose (16.2 mm), D-xylose (16.2 mm), N-acetyl-D-galactosamine (9.3 mm) and inositol (4.6 mm). To obtain proteins with the highest lectin activity of neurons and glial cells, the neurolectins were purified by affinity chromatography on a column of tris-acryl-inositol and tris-acryl-galactose. Inositol-specific neurolectin was isolated from the enriched fractions of glial cells (GL-I), and the neurolectin specific to galactose was isolated from the enriched fractions of neurons (NL-GAL). The molecular weights of the neurolectins were measured using an HPLC system (Millipor-Waters, USA). Their molecular weights, respectively, were GL-I = 11.5 kDa, and NL-Gal = 13.5 kDa. Both lectins were glycoproteins containing carbohydrates 28% and 26%, respectively.

It should be noted that NL-Gal neurolectin contains the following amino acids: leucine, isoleucine, valine, phenylalanine, tryptophan, glutamic acid, threonine, glycine, serine, glutamine, asparagine and arginine, whereas in GL-I, with the exception of these amino acids, in addition, proline and tyrosine were found. The presence of SH groups was noted in both neurolectins, which was established by the Ellman method [23]. The number of SH groups per mg / protein was 35×10^{-4} M for NL-Gal, while 15×10^{-4} M for GL-I. Both neurolectins showed particular sensitivity to Ca^{+2} ions and were most inhibited in the presence of 0,2 mM Ca^{+2} EGTA chelators.

It is noteworthy that the neurolectins of glial cells of the embryo are predominantly presented in soluble form, which is usually characteristic of the brain of embryonic animal cells. It is more likely that the soluble forms of neurolectins in the process of postnatal development turn into a bound form.. It is worth noting that endogenous neurolectin was also found in Schwan cells. It has been suggested that Schwan cells neurolectins are involved in the stabilization of compact myelin [11, 12].

Taking into account the strategic role of glial cells in providing trophic function of cells, an attempt was made to study the effect of amino acids, neurotransmitters and biologically active substances on the hemagglutination of trypsinized rabbit erythrocytes [14, 20, 22], suggesting that the results of these experiments should give an answer about the possible role of neurolectins in capture and their inactivation. Using the hapten-inhibitor method, it has been experimentally shown that the hemagglutination activity of trypsinized rabbit erythrocytes NL-Gal is inhibited by arginine (37.5 mm), L-ser-

ine (18.7 mm), L-glutamine (18.7 mm), glycine (0.31 mm), hemagglutination of neurolectins GL-I is inhibited by L-arginine (37.5 mm), L-serine (18.7 mm), L-glutamine (9.4 mm), tyrosine (37, 5 mM), acetylcholine (0.62 mm), beta-alanine (0.156 mm), serotonin (0.32 mm), epinephrine (1.25 mm), norepinephrine (0.62 mm), dopamine (0.156 mm), tyramine (0.63 mmol), hydroxytyramine (0.156 mmol), choline bromide (0.08 M) and hydroxytryptamine (1.25 mM). It is important to note that a number of other biologically active substances had also an inhibitory effect on the activity of neurolectin GL-I, but with relatively high concentration: gamma-aminobutyrate (> 100 mM) and methoxytyramine (> 100 mM). The quotation marks provide data on the concentration of hemagglutination inhibitors [24-26].

Based on the presented material, the determination of the orientation of the active center of GL-I and NL-Gal on cell membranes was of great interest. It has been shown that native glial cells cause agglutination of trypsinized rabbit erythrocytes. GL-I agglutination is completely inhibited by inositol (0.6 mM), which suggests that the GL-I active center on the membrane of glial cells is oriented to the outer side of the membrane in the direction of the intercellular space and the active center of NL-Gal is oriented towards the cell cytoplasm [7, 14, 16].

The question arises: what role can the neurolectins play in the trophic function of glial cells. After the hapten-inhibitory method established that biologically active substances, neurotransmitters and amino acids had an inhibitory effect on agglutination of trypsinized rabbit erythrocytes, it was concluded that GL-I neurolectin can actively participate in the transport of neurotransmitters, amino acids and biologically active substances and their metabolism and inactivation. It was previously shown that under conditions of excess neurotransmitters in the neuron-glia-synapse region by glial cells and synaptosomes, active absorption and detoxification of acetylcholine, serotonin, dopamine, and gamma-aminobutyric acid occurs [24, 25]. It should be noted that the absorption of serotonin by glial cells and their inactivation occurs actively by conjugation with glucuronic acid, resulting in the formation of biologically inactive glucuronideserotonin [26]. The absorption of dopamine and serotonin by glial cells of the rabbit cerebral cortex also occurs in the same way.

Thus, it has been found that neurolectins are mainly presented in nerve cells, subcellular fractions, pathways, dendritic basement membranes, nerve endings, and synaptic vesicles. It is interesting that the number of neurolectins and their activity are mainly observed during mass synaptogenesis, which indicates their active participation in the formation of synapses. Therefore, naturally, the researches were begun to study the distribution and function of neurolectins at the level of synaptic vesicles, in connection with their possible role in secretion and accumulation of neurotransmitters in synaptic vesicles, which were isolated according to the method of De Robertis and his colleagues [27].

In 2002 and 2004, inositol-specific lectin was isolated from synaptic vesicles of chicken and bovine brain (BVL-I) with Triton X-100 solution [28, 29], which turned out to be a glycoprotein with a total content of 10% carbohydrates. Neurolectin showed a high sensitivity to Ca^{+2} ions, in the presence of 0.2 mM concentration of Ca^{+2} chelator of the EGTA, the activity of neurolectins was maximally inhibited. Of particular interest is the fact that the neurolectin BVL-I exhibits a specific affinity for phosphatidylinositol and phosphatidylcholine and is localized on the outer surface of the vesicular membrane, which has been experimentally proven. It has been first discovered that agglutination of trypsinated rabbit erythrocytes occurs under the influence of bovine brain native synaptic vesicles, while an agglutination induced by synaptic vesicles is inhibited by inositol [8, 20, 29]. This suggests that BVL-I is an inositol-specific neurolectin. Neurolectin is relatively rich in sulfur-containing amino acids, the amount of which is 21.4×10^4 M per 1 mg of protein.

On the other hand, neurolectins from synaptic vesicles were also seen from the brains of mature chickens. For maximum extraction of neurolectins, 0.5% Triton X-100 on 40 mM PPB (pH 7.4) was used. As a result of using this solution, specific inositol (SVL-I) and N-acetyl-D-galactosamine (SVL-NAGA) were isolated and purified on a column of immobilized Sepharose 4B N-acetyl-D-glucosamine and inositol. The lectin activity of the extracted protein increased by 2–5 times. Both lectins are glycoproteins containing 6% and 13% carbohydrates, respectively. Neurolectins are enriched with sulfur-containing amino acids, the average amount of which is 21.4×10^4 M per 1 mg of protein.

Neurolectin SVL-I is highly sensitive to Ca^{+2}, and therefore, its activity is completely inhibited by Ca^{+2} with the EGTA chelator. Its location and orientation in the vesicles were also of great interest. In order to resolve this question, it was necessary to clarify the effect of SVL-1 and SVL-NAGA on agglutination of trypsinized rabbit erythrocytes. It

was found that native synaptic vesicles agglutinate trypsinized rabbit erythrocytes. The agglutination is completely inhibited by specific hapten-inositol and phosphatidylinositol (0.03 mg/100 μl) [25, 29-32]. It becomes apparent that the neurolectins BML-I and VL-I possess essentially the same characteristics and are characterized by the same affinity for haptens, namely, inositol, inositol phospholipid and phosphatidilcholine.

Based on these data, a hypothesis is suggested that SVL-1 and SVL-NAGA participate in the secretion of neurotransmitters from synaptic vesicles [6, 8, 33,34]. According to the proposed hypothesis, neurolectin SVL-1 is fixed on the presynaptic membrane in the form of a terminal carbohydrate residue of inositol and inositol phospholipid, which is oriented towards the cytoplasm of the vesicles. At the same time, the presynaptic membrane and synaptic vesicles merge, forming a vesicular lumen, through which an active influx of Ca^{+2} ions from the synaptic cleft occurs. The formation of a similar structure was shown in the electron microscopic studies. The formation of a presynaptic lumen is accompanied by the secretion of neurotransmitters, while the stimulation of receptors activates the phospholipase C enzyme.. As a result of the action of phospholipase C, inositol phospholipid is cleaved into inositol-1,4,5-triphosphate (ITP) and diacylglycerol. The latter remains in the presynaptic membrane, while ITP in bound form with the vesicles is separated from the membrane. A part of ITP in the free state promotes an increase in the concentration of Ca^{+2} ions in the cytoplasm, and diacylglycerol activates protein kinase C. Therefore, with an increase in the concentration of cytoplasmic Ca^{+2}, the output of neurotransmitters from synaptic vesicles through the presynaptic lumen is accelerated (Fig.). This completes the secretion of the neurotransmitter and begins the stage of formation of synaptic vesicles and their filling with neurotransmitters.

As for the formation and closure of the lumen of synaptic vesicles, in accordance with the proposed scheme, lectin SVL-1, SVL-NAGA and Ca^{+2} ions are involved in it. With an increase in the concentration of Ca^{+2} ions, the inhibition of ITP binding to receptors in the cytoplasm is observed, and at the same time, the ligand-receptor bond (ITP-SVL-1) is destroyed. This process is enhanced with the splitting of ATP by neurolectins and the accumulation of phosphate around the vesicles [35-38]. It is well known that the binding of neurolectin to receptors is disrupted in an acidic environment [34]. Free-form ITP stimulates the release and accumulation of Ca^{+2} ions in the cytoplasm. As a result, an agglutination and fusion of vesicular membranes are enhanced, and neurolectin VL-NAGA is actively involved in the process. It should be noted that VL-NAGA is more sensitive to Ca^{+2} ions (3 mM) compared to VL-I (> 100 mM). Ca^{+2} ions contribute to the fusion of the ends of both vesicular and presynaptic membranes. Thus, it is concluded that after the release and loading of vesicles with neurotransmitters, fusion and union of the vesicular end membranes is ensured. It is assumed that the hypothesis needs a further development of the biochemical foundations of the individual stages, but the opinion about the participation of neurolectins in the secretion of neurotransmitters and membrane fusion, in all likelihood, will not cause doubt.

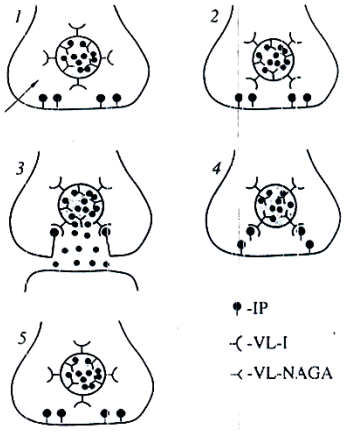

Fig. *Hypothetical scheme for the participation of the neurolectins VL-1 and VL-NAGA in the secretion of neurotransmitters.*

1 - The synapse is at rest. 2 - Antibody vesicular lectin SVL-1 on the presynaptic membrane with inositol phospholipid. 3 - The formation of presynaptic lumen and the secretion of neurotransmitters. 4 - Separation of vesicles from inositol phospholipid (IP) and restoration of the presynaptic membrane. 5 - Complete restoration of the synaptic vesicle in a relaxed state. Designations: IP - inositol-triphosphate, SVL-1 - vesicular inositol-specific neurolectin, SVL-NAGA - vesicular N-acetyl-D-galactosamine-specific neurolectin

Neurolectins from mitochondria

Since the quantitative distribution of neurolectins in the animal brain was established, it was of great interest to study the distribution of neurolectins at the level of subcellular fractions of nerve

cells. Neurolectin was extracted from brain mitochondria with a specially selected 0.5% solution of X-100 Triton and purified under conditions of 80% saturation with ammonium sulfate. It was found that agglutination with mitochondrial neurolectin is specifically inhibited by glucose, galactose, N-acetylglucosamine and N-acetyl-galactosamine hence was called BML-GluG [39]. BML-GluG was fractionated on a Sephadex G-100 column. Chromatography revealed isoforms of BML-GluG neurolectin (A, B), which showed lectin activity. BML-GluG is a glycoprotein containing carbohydrates at a concentration of 16.7 μg / mg protein. Neurolectin also contained disulfide groups. After restoration of disulfide bonds by dithiothreitol, lectin activity was maximally inhibited. BML-GluG was found to be sensitive to Ca^{+2} ions. In the presence of Ca^{+2} EGTA chelator, at a concentration of 0.2 mM, an agglutination of trypsinized rabbit erythrocytes was maximally inhibited. It was necessary to study its orientation on the mitochondrial membrane. For this purpose, it has been found that the agglutination of trypsinized rabbit erythrocytes occurs by the homogenate of native mitochondria and, therefore, using the hapten-inhibitory method, it is shown that agglutination of trypsinized rabbit erythrocytes using BML-GluG was maximally inhibited by N- acetyl-D-glucosamine. Thus, it is concluded that BML-GluG is oriented outside the mitochondrial member [39].

Nuclear neurolectins of rat brain cell

Data on nuclear neurolectins of nerve cells are quite limited. Therefore, special attention was paid to the nuclear neurolectins of the rat brain [40-42]. In order to establish the presence of neurolectins in the nuclei of rat brain nerve cells, it has been previously established that agglutination of trypsinized rabbit erythrocytes occurs with a native nuclear homogenate. In the agglutinated supernatant, the fractional composition of the proteins was studied by electrophoresis in a 7.5% polyacrylamide gel. In contrast to electrophoregrams of a non-agglutinated nuclear supernatant, a number of protein fractions were absent in the agglutinated supernatant, which indicated that as a result of the binding of nuclear proteins during agglutination with trypsinized rabbit erythrocytes, a number of protein supernatant fractions were not found. Based on these experimental data, a study was begun on the distribution of neurolectins in the cell nuclei of rat brain. For this, various composition buffers were tested, among which the buffer of the following composition turned out to be the most promising: 0.5% Triton X-100 + 0.9% NaCl + 20 mM potassium phosphate buffer (PPB), pH 5.0 [43, 44]. After homogenization of the nuclear fraction, soluble proteins were removed using PPB, the mixture was dialyzed against hemagglutination buffer, and the lectin activity of the mixture was determined. It has been shown that the specific activity of nuclear neurolectin is 104.4 units. Subsequently, the cell nucleus neurolectin was purified by the method of stepwise salting out with ammonium sulfate at a saturation of 20%, 40%, 80%. These proteins were purified by HPLC (Waters, USA) on Protein PAK-300-SW gel tandem filter columns. It was found that, when saturated, under conditions of 40% saturation with ammonium sulfate, the specific neurolectin activity increased one and a half times. After fractionation of the proteins on glutaraldehydefixed tripressed blue rabbit erythrocytes, the specific neurolectin activity of the nuclei (Nuc-L) increased 5 times and reached 534 units. It should be noted that nuclear neurolectins during gel filtration on a Protein PAK-300-SW column are divided into two protein fractions. Both fractions of proteins are characterized by lectin activity. Using the hapten-inhibitory method, it was shown that both neurolectins of the nucleus of the brain of the rat haptens were N-acetyl-D-glucosamine, D-galactose and L-fructose.

Conclusion

Quantitative and qualitative distribution of neurolectins in brain neurons, glial cells, synapses, and their subfractions (mitochondria, cell nuclei) and their proper hapten have been established in animals (rats, chickens, bulls). The possible role of neurolectins secreted by neurons, glial cells, and synaptosomes is given depending on trophic function and biologically active substance absorption and inactivation depending on the orientation of the membrane neurolectins.

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