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An endogenous inhibitor protein of synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase

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An inhibitor protein of synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase was purified to apparent homogeneity from rat cerebrum by a molecular weight cut followed by chromatography of cytosol proteins with molecular weights between 10000 and 3500 on DEAE-Sephadex at pH 5.2. The inhibitor could be partially inactivated by proteinases and dithiothreitol, but was heat-stable. Gel filtration gave a molecular weight of about 6000. Like the ($Ca^{2+} + Mg^{2+}$)-ATPase inhibitor protein isolated from erythrocytes, the inhibitor from brain contains a characteristic high proportion of glutamic acid (36%) and glycine (37%) residues. Synaptic plasma membrane Mg^{2+}-ATPase and microsomal membrane ($Ca^{2+} + Mg^{2+}$)-ATPase did not respond to the inhibitor. Synaptic plasma membrane and erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPases, however, were affected. Inhibitory influence on synaptic membrane ($Ca^{2+} + Mg^{2+}$)-ATPase was reversible, since inhibition could be relieved upon removal of inhibitor from saturable sites on the membrane. The inhibitor is not a calmodulin-binding protein, since the concentration of calmodulin for half-maximal activation of the ATPase was unaffected by its presence. Mode of inhibition of the ($Ca^{2+} + Mg^{2+}$)-ATPase by the inhibitor was non-competitive.

Introduction

Calcium is important in the control of neuronal excitability and transmitter release [1–4]. Various mechanisms have been suggested to be involved in the regulation of intraneuronal free calcium ion concentration. These include an exchange of intraneuronal calcium with extracellular sodium via a Na^+-Ca^{2+} antiport process [5–7], transport of the ion into mitochondria [8,9], binding by intraneuronal and membrane proteins [10,11] and calcium extrusion by Ca^{2+}-stimulated Mg^{2+}-dependent ATPases [8,12–15].

Two high-affinity Ca^{2+}-stimulated Mg^{2+}-dependent ATPases, one associated with synaptic plasma membrane and the other with microsomal membrane, have been suggested to be involved in calcium transport across neuronal membranes [16]. Based on their differential sensitivity to vanadate, the synaptic plasma membrane enzyme is considered to be similar to that of the erythrocyte and sarcolemmal enzymes, while the microsomal membrane ATPase is similar to the Ca^{2+}-ATPase of sarcoplasmic reticulum.

Activity of the erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPase is known to be regulated by protein modulators. The calcium-binding protein, calmodulin, plays the part of a protein activator of the transport enzyme [17,18]. Its action is, however, opposed by an inhibitor protein of the ATPase first reported to be present in erythrosol by Au [19] and later confirmed by Wüthrich [20] and by Lee and Au [21]. This ($Ca^{2+} + Mg^{2+}$)-ATPase inhibitor protein has a molecular weight

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of 7500. It is not a calmodulin-binding protein but has a direct inhibitory action on the erythrocyte membrane enzyme. Since synaptic plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase and erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase are thought to be similar enzymes, it thus appears desirable to search for the presence of a synaptic plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase inhibitor protein in brain. Such an inhibitor, if present, would be expected to play an important role, along with calmodulin, in the control of $(Ca^{2+} + Mg^{2+})$-ATPase involved in the regulation of intraneuronal calcium level.

Materials and Methods

Materials

Vanadium-free ATP and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO). PM-10 and YM-2 ultrafiltration membranes were products of Amicon (Tokyo). Calmodulin was obtained from Pharmacia Fine Chemicals (Uppsala). Dialysis membranes (molecular weight cutoff at 3500) were from Arthur H. Thomas Co. (Philadelphia, PA). These membranes were boiled with EDTA and thoroughly rinsed with deionised water before use. Boiling was absolutely essential to remove a contaminating factor that could also inhibit membrane $(Ca^{2+} + Mg^{2+})$-ATPase.

Methods

Purification of $(Ca^{2+} + Mg^{2+})$-ATPase inhibitor protein from rat brain. All isolation procedures were conducted at 0–4°C. In a typical preparation, 90 male Sprague-Dawley rats of 200–250 g body weight were decapitated, the forebrains were rapidly removed of blood capillaries, washed with 0.27 M sucrose/0.5 mM phenylmethylsulfonyl fluoride/1 mM Tris (pH 7.4, 298 mosM) and homogenized in 4 vol. of the same buffer by a Potter-Elvehjem Teflon/glass homogenizer. The homogenate was centrifuged at $100\,000 \times g$ for 1 h. NaCl solution was then added slowly with gentle stirring to the supernatant (cytosol fraction) to give a final concentration of 0.5 M. The addition of salt facilitated the release of protein-bound inhibitor [21]. The treated cytosol fraction was subjected to ultrafiltration through an Amicon PM-10 membrane. The filtrate thus obtained was then concentrated 6-fold by ultrafiltration through a YM-2 membrane. The concentrate was dialyzed against 50 mM imidazole-HCl (pH 5.2) and 40 ml of the dialyzed concentrate were eluted with the same buffer on a DEAE-Sephadex A-25 column (2.6×30 cm) at a flow-rate of 35 ml/h. After unbound proteins were removed, a linear NaCl gradient of 0–0.6 M was applied. Fractions of 7.5 ml were collected. Each protein peak was separately concentrated 20–25-fold, dialyzed against 5 mM imidazole-HCl (pH 7.4) before being assayed for $(Ca^{2+} + Mg^{2+})$-ATPase inhibitory activity.

Inhibitor protein was also purified to apparent homogeneity from adult pig erythrocytes by the method of Lee and Au [21], except that the last electrophoresis step was substituted by rechromatography on a second DEAE-Sephadex A-25 column equilibrated with 50 mM imidazole-HCl (pH 5.2) containing 0.14 M NaCl. A linear NaCl gradient of 0.14–0.25 M lasting for 16 h was then applied. Protein eluted between 0.163 and 0.170 M NaCl was collected, concentrated by ultrafiltration through the YM-2 membrane then dialyzed against 5 mM imidazole-HCl (pH 7.4) before being assayed for Ca^{2+}-pump ATPase inhibitor activity. Yield of inhibitor protein was 0.25 mg protein/l cells.

Preparation of synaptic plasma membranes and erythrocyte membranes. Synaptic plasma membranes were prepared from rat brain by the method of Hakim et al. [22], while erythrocyte membranes were prepared from pig red cells by the method of Lee and Au [21]. Both membranes were stripped of calmodulin so that response of membrane-bound $(Ca^{2+} + Mg^{2+})$-ATPase to inhibitor protein could be studied without interference.

ATPase assays. ATPase activity was measured at 37°C as ADP production which was linked to oxidation of NADH and monitored continuously at 366 nm. The assay medium contained 20 mM NaCl/100 mM KCl/6 mM $MgCl_2$/6 mM ATP/0.1 mM ouabain/0.3 μg oligomycin/0.2 mM NADH/0.5 mM phospho*enol*pyruvate/1 unit each of pyruvate kinase and lactate dehydrogenase/50 mM imidazole (pH 7.4) in a final volume of 1 ml. When $(Ca^{2+} + Mg^{2+})$-ATPase activity was measured, 1.8 mM $CaCl_2$ and 2 mM EGTA were included. Mg^{2+}-ATPase, on the other

hand, was assayed in the presence of 2 mM EGTA but no addition of Ca^{2+}. (Ca^{2+} + Mg^{2+})-ATPase activity is defined as the activity measured in the presence of Ca^{2+} minus the activity measured in its absence. ATPase activity was expressed as μmol/h per mg membrane protein. Both synaptic plasma membranes and microsomes possessed (Ca^{2+} + Mg^{2+})-ATPase activity as was observed by Michaelis et al. [16]. In the assay of synaptic plasma membrae (Ca^{2+} + Mg^{2+})-ATPase, activity measured in the presence of 6 μM sodium orthovanadate was subtracted from total (Ca^{2+} + Mg^{2+})-ATPase activity.

When studying the effect of the inhibitor protein on ATPase, the inhibitor (about 2 μg protein) was preincubated with the synaptic plasma membrane preparation (50 μg protein) for 50 min at 27°C before starting the reaction with addition of ATP.

Erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase activity was similarly measured. The assay medium, however, contained 80 mM NaCl/28 mM KCl/3.6 mM $MgCl_2$/0.2 mM $CaCl_2$/0.1 mM EGTA/2.5 mM ATP/0.1 mM ouabain/0.2 mM NADH/0.5 mM phospho*enol*pyruvate/1 unit each of pyruvate kinase and lactate dehydrogenase/80 mM histidine adjusted to pH 8.0 with NaOH. Preincubation of inhibitor protein with erythrocyte membranes was at 37°C for 1 h.

Amino acid analysis of inhibitor proteins. Purified ATPase inhibitor protein from either erythrocytes or brain was hydrolyzed in vacuo using constant-boiling HCl (Pierce Chemical Co., Rockford, IL) at 110°C for 24, 48 and 72 h. Serine and threonine values were extrapolated to zero hydrolysis time, while valine value was taken from a hydrolysis time of 72 h. Cysteine was determined as cysteic acid; methionine and tryptophan were determined after hydrolysis with 4 M methanesulfonic acid (Pierce Chemical Co.). The amino acids were determined on a LKB 4400 amino acid analyzer according to the instructions given by the manufacturer.

Protein determination. Membrane protein determination was by the method of Lowry et al. [23] using bovine serum albumin as standard. With the inhibitor, however, protein determination was by quantitative amino acid analysis.

Results

Synaptic plasma membrane (Ca^{2+} + Mg^{2+})-ATPase inhibitor protein was purified to apparent homogeneity from rat cerebrum by subjecting the cytosol fraction to molecular weight cuts followed by chromatography of proteins with molecular weights between 10000 and 3500 on DEAE-Sephadex at pH 5.2. The inhibitor was eluted as a major protein peak by 0.22–0.23 M NaCl from the anion-exchange column (Fig. 1). Contamination of the brain inhibitor by inhibitor derived from remaining erythrocytes was estimated to be not more than 0.004%.

That the inhibitor is macromolecular is suggested by its molecular weight of 6000 as determined by gel filtration. Its protein nature is suggested by its sensitivity towards proteinases, though it is rather heat-stable (Table I). The amino acid composition of the purified brain inhibitor was found to be strikingly similar to that of the protein inhibitor derived from pig erythrosol. Both contained a characteristic high proportion of

TABLE I

CONDITIONS AFFECTING THE ACTIVITY OF SYNAPTIC PLASMA MEMBRANE (Ca^{2+} + Mg^{2+})-ATPase INHIBITOR PROTEIN

Synaptic plasma membrane (Ca^{2+} + Mg^{2+})-ATPase activity was 4.2 μmol/h per mg membrane protein. The inhibitor protein (1.8 μg protein) suppressed ATPase activity of the membranes (50 μg protein) by 57%. Mg^{2+}-ATPase and microsomal (Ca^{2+} + Mg^{2+})-ATPase activities, however, were unaffected. Proteinase treatment was performed by incubating the inhibitor protein for 1 h at 37°C with the proteinase (2 μg protein) in the presence of 0.1 mM $CaCl_2$ (pH 7.4). Digestion was stopped by 30 μM phenylmethylsulfonyl fluoride and the digested inhibitor was assayed for inhibitor activity.

Treatment of inhibitor	% Untreated activity
Untreated	100
Heating at 100°C	
6 min	100
12 min	90
Trypsin	66
Chymotrypsin	67
Dithiothreitol	
1 mM	69
2 mM	58
4 mM	58

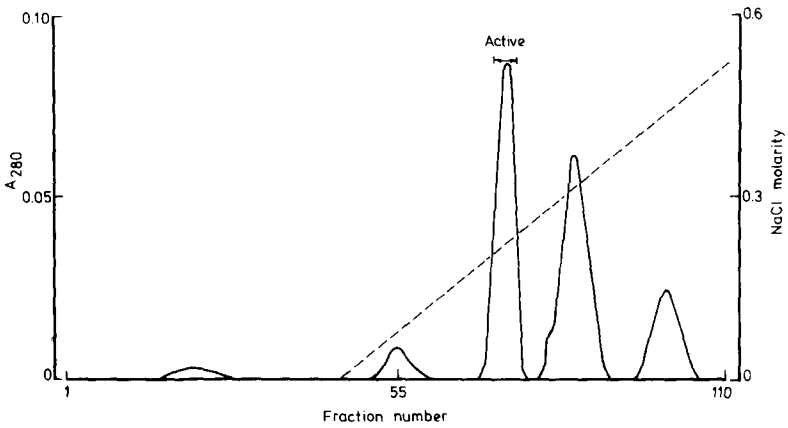

Fig. 1. Purification of synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase inhibitor protein by DEAE-Sephadex chromatography. 40 ml YM-2 concentrate, derived from cytosol fraction, were eluted with 50 mM imidazole-HCl (pH 5.2) and subsequently with a linear gradient of 0–0.6 M NaCl. Absorbance was measured at 280 nm (———). The active fractions are marked.

glutamic acid (brain inhibitor, 36%; erythrocyte inhibitor, 38%) and glycine residues (brain inhibitor, 37%; erythrocyte inhibitor, 35%). Both contained a similar amount of cysteine (brain inhibitor, 7%; erythrocyte inhibitor, 5%) and aspartic acid residues (brain inhibitor, 3.5%; erythrocyte inhibitor, 5%), but both were found to have no detectable proline, isoleucine, tyrosine, phenylalanine, histidine, lysine and arginine residues. The presence of a high proportion of acidic residues in the inhibitor proteins (isoelectric pH of red cell inhibitor = 4.8) explains why salt was required to elute them from the anion-exchanger equilibrated at pH 5.2.

Intact disulfide bridges are essential for inhibitor activity. This is deduced from the finding that dithiothreitol could substantially decrease the activity of the brain inhibitor (Table I). In comparison, red cell ($Ca^{2+} + Mg^{2+}$)-ATPase inhibitor protein activity is actually more dependent on intact disulfide bridges, since 2 mM dithiothreitol was found to decrease its inhibitory activity by 74%. Glutathione, at the same concentration, could in fact abolish red cell inhibitor activity completely.

Inhibitor protein isolated from rat brain was found to have the ability to suppress erythrocyte membrane ($Ca^{2+} + Mg^{2+}$)-ATPase activity, while the red cell inhibitor protein in turn could inhibit synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase. Similarities between inhibitor proteins and ($Ca^{2+} + Mg^{2+}$)-ATPase from the two sources are thus suggested.

With reference to its action on brain membrane enzymes, however, the synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase inhibitor protein isolated from rat cerebrum is specific, since it was found to have no action on the activities of synaptic plasma membrane Mg^{2+}-ATPase and microsomal membrane ($Ca^{2+} + Mg^{2+}$)-ATPase. Moreover, K_m (ATP) = 5.8 μM and $K_{0.5}$ (calmodulin) = 0.31 nM determined for the microsomal membrane ATPase were found to be unaffected by the inhibitor.

The mode of inhibition of synaptic plasma

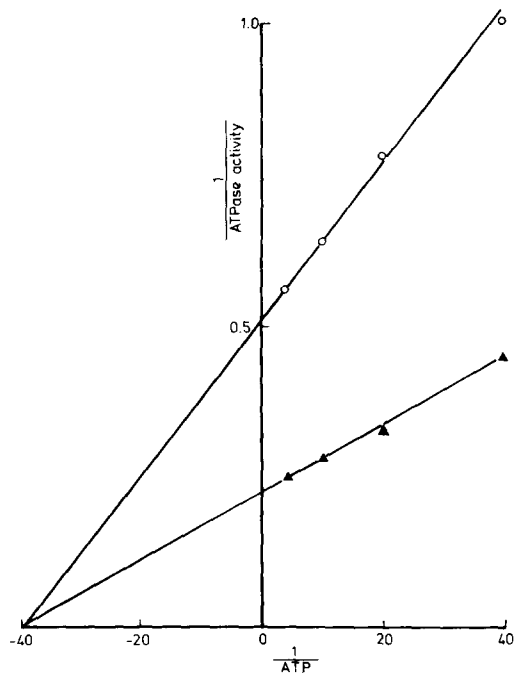

Fig. 2. Lineweaver-Burk plot for synaptic plasma membrane ($Ca^{2+} + Mg^{2+}$)-ATPase reaction in the absence (▲———▲) and presence (○———○) of inhibitor (2 μg protein). ATP (mM); ATPase activity (μmol/h per mg membrane protein).

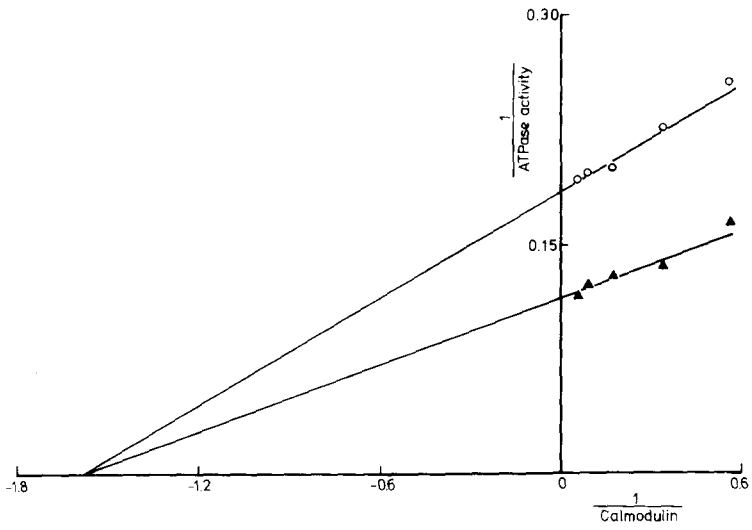

Fig. 3. Double-reciprocal plot of synaptic plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol/h per mg membrane protein) versus calmodulin concentration (nM) for determination of the concentration of calmodulin giving half-maximal activation of the enzyme. ▲——▲, Calmodulin only; ○——○, calmodulin and inhibitor (2 μg protein).

membrane $(Ca^{2+} + Mg^{2+})$-ATPase by the protein inhibitor isolated from brain was non-competitive, with K_m (ATP) remaining unchanged at 25 μM, but a 56% decrease of V from 4.55 to 1.98 μmol/h per mg membrane protein (Fig. 2). Inhibition of the ATPase was thus not a result of a decrease in the affinity of the enzyme for its substrate, ATP.

The $(Ca^{2+} + Mg^{2+})$-ATPase inhibitor protein is not a calmodulin-binding aprotein. This is because calmodulin-depleted membranes were used for assay of inhibitor activity, and thus inhibition, if observed, could not be due to the calmodulin-binding protein. Besides, the concentration of calmodulin for half-maximal activation of synaptic plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPase remained unchanged at 0.63 nM inspite of the presence of the inhibitor (Fig. 3). More calmodulin would be required for enzyme activation if the inhibitor is a calmodulin-binding protein-like molecule.

The effect of the protein inhibitor on the synaptic plasma membrane ATPase was reversible (Table II), since inhibition could be relieved by removal of the inhibitor from saturable sites on the membranes (Fig. 4). It is therefore unlikely that the inhibitor functioned as a proteinase to bring about irreversible degradation of the ATPase. Detachment of the inhibitor protein from synaptic plasma membranes required 0.6 M NaCl and 0.1 mM EGTA. Ionic bonds and Ca^{2+} may thus be involved in the binding of the $(Ca^{2+} + Mg^{2+})$-ATPase inhibitor protein to synaptic membranes. Inhibitory action of the red cell inhibitor protein on erythrocyte membrane $(Ca^{2+} + Mg^{2+})$-ATPase was also found to be reversible and detachment of the inhibitor from red cell membranes required 2 mM EDTA in isotonic NaCl (results not shown).

TABLE II

REVERSIBLE INHIBITION OF SYNAPTIC PLASMA MEMBRANE $(Ca^{2+} + Mg^{2+})$-ATPase BY INHIBITOR PROTEIN

Synaptic plasma membranes were incubated with inhibitor protein at 27°C for 50 min and the membranes were either washed with 5 mM imidazole-HCl (pH 7.4) or extracted with the same volume of the buffer containing 0.1 mM EGTA/0.6 M NaCl for 5 min at 4°C to remove membrane-bound inhibitor. The extracted membranes were then washed three times with 10 mM Tris-citrate (pH 7.4) to remove EGTA and NaCl. Control membranes with and without preincubation with inhibitor protein were similarly treated. All membrane preparations were adjusted to the same protein concentrations (50 μg) before they were assayed for $(Ca^{2+} + Mg^{2+})$-ATPase activity (μmol/h per mg membrane protein).

Membranes	$(Ca^{2+} + Mg^{2+})$-ATPase activity	%Inhibition of ATPase
No inhibitor added	4.02	-
Inhibitor added	1.92	52.2
Inhibitor added but extracted	3.99	0.75

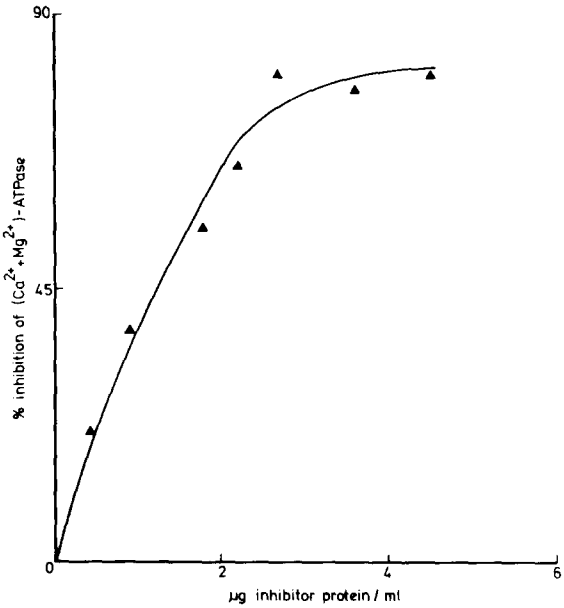

Fig. 4. Saturation of synaptic plasma membrane (Ca^{2+} + Mg^{2+})-ATPase sites by inhibitor protein. Basal (Ca^{2+} + Mg^{2+})-ATPase activity of membranes (50 μg protein) was 4.5 μmol/h per mg membrane protein. Sites on synaptic plasma membrane (1 mg protein) were saturated by about 0.01 μmol inhibitor protein.

Discussion

The present study represents the first successful attempt in the isolation and characterization of a specific (Ca^{2+} + Mg^{2+})-ATPase inhibitor protein in a non-erythrocyte tissue, namely, the brain. Owing to its specific action on synaptic plasma membrane (Ca^{2+} + Mg^{2+})-ATPase and not on microsomal membrane (Ca^{2+} + Mg^{2+})-ATPase, the inhibitor protein could be employed as a tool for differentiating the two ATPases, which are both thought to be involved in Ca^{2+} transport across membranes of brain neurons [16]. In fact, the (Ca^{2+} + Mg^{2+})-ATPase inhibitor protein is also positively identified in rat epididymal fat pad, though its presence in cardiac muscle was questionable (Au, K.S., unpublished data). Search for the presence of the inhibitor protein among other tissues, for example, intestine, thyroid and lymphocytes, which are known to possess membrane-bound (Ca^{2+} + Mg^{2+})-ATPase activity [24–26], is expected to be fruitful. Moreover, in view of the many similarities described here for the synaptic plasma membrane (Ca^{2+} + Mg^{2+})-ATPase inhibitor protein and erythrocyte membrane (Ca^{2+} + Mg^{2+})-ATPase inhibitor protein, it is tempting to suggest that they are very similar molecules, allowing for tissue and species differences. Work is now in progress to identify the involvement of the brain inhibitor protein in Ca^{2+}-dependent neuronal functions and to see if the inhibitor protein has an ubiquitous distribution.

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