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Molecular characterization of a two-domain form of the neuronal voltage-gated P/Q-type calcium channel $\alpha_1$2.1 subunit

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Abstract **We characterized the neuronal two-domain (95kD-$\alpha_1$2.1) form of the $\alpha_1$2.1 subunit of the voltage-gated calcium channels using genetic and molecular analysis. The 95kD-$\alpha_1$2.1 is absent in neuronal preparations from CACNA1A null mouse demonstrating that $\alpha_1$2.1 and 95kD-$\alpha_1$2.1 arise from the same gene. A recombinant two-domain form (α_{1AI-II}) of $\alpha_1$2.1 associates with the β subunit and is trafficked to the plasma membrane. Translocation of the α_{1AI-II} to the plasma membrane requires association with the β subunit, since a mutation in the α_{1AI-II} that inhibits β subunit association reduces membrane trafficking. Though the α_{1AI-II} protein does not conduct any voltage-gated currents, we have previously shown that it generates a high density of non-linear charge movements [Ahern et al., Proc. Natl. Acad. Sci. USA 98 (2001) 6935–6940]. In this study, we demonstrate that co-expression of the α_{1AI-II} significantly reduces the current amplitude of $\alpha_1$2.1/β_{1a}/$\alpha_2\delta$ channels, via competition for the β subunit. Taken together, our results demonstrate a dual functional role for the α_{1AI-II} protein, both as a voltage sensor and modulator of P/Q-type currents in recombinant systems. These studies suggest an in vivo role for the 95kD-$\alpha_1$2.1 in altering synaptic activity via protein–protein interactions and/or regulation of P/Q-type currents.**

Key words: 95kD-$\alpha_1$2.1; Two-domain subunit; Episodic ataxia type 2; Calcium channels

1. Introduction

High voltage-gated calcium channels play important roles in intracellular and intercellular communication. The channels allow Ca^{2+} influx into the cells in response to depolarization and hence serve as transducers of electrical information into cellular signals resulting in muscle contraction, hormone secretion, neurotransmitter release and regulation of gene transcription [2,3]. Different classes of high voltage-gated channels (L, N, R and P/Q) have been defined on the basis of their biophysical characteristics and sensitivity to pharmacological agents and serve specialized roles [3] in their tissue of expression. The P/Q- and the N-type channels predominantly function in neurotransmitter release at central synapses [4] and the neuromuscular junction [5], while the L-type channels function in excitation–contraction coupling in the skeletal and cardiac muscles [6,7].

At the molecular level, these channels are heteromultimers of different subunits composed of at least the α_1, $\alpha_2\delta$ and β subunits [8–10]. In addition, the skeletal muscle channel also contains a γ_1 subunit [11] and recently other γ subunits have been identified [12–14]. The γ_2 and γ_3 subunits are associated with the neuronal channels [15], suggesting that the four-subunit complex is a common feature of the high voltage-gated Ca^{2+} channel composition.

The α_1 subunit, a large hydrophobic protein with 24 transmembrane regions distributed over four domains, forms the voltage sensor and the pore of the channel to allow Ca^{2+} influx into the cell in a voltage-dependent manner. The other subunits are auxiliary and serve to modulate the trafficking and the biophysical properties of the channel [2]. Alternative forms of each of the Ca^{2+} channel subunits arise by a variety of mechanisms including splicing, editing and post-translational modifications of the protein [16–18]. These variant forms contribute to the diversity of biophysical properties and functions for the channels.

Previous studies on purified channel preparations and synaptic plasma membranes from rabbit brain have demonstrated the existence of a 95 kDa two-domain form of the $\alpha_1$2.1 subunit [19]. This protein is predicted to be composed of the amino terminus, the first two transmembrane domains and a part of the II–III loop of the $\alpha_1$2.1 subunit [19]. The 95kD-$\alpha_1$2.1 protein is enriched in synaptic membranes, is glycosylated and binds to the β subunit. In addition to the rabbit brain, this protein is expressed in the Sertoli cells of the testes [20]. However, the role of the protein in vivo and the nature of its origin remain unknown.

In this study, we demonstrate that the 95kD-$\alpha_1$2.1 is enriched in the rabbit cerebellum. Further, using a mouse that is genetically targeted to ablate the CACNA1A gene, we show that both the full-length $\alpha_1$2.1 and the truncated 95kD-$\alpha_1$2.1 proteins arise from the same gene. Moreover, we demonstrate the ability of a similar recombinant protein (α_{1AI-II}) to form a

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complex with the β subunit and traffic to the plasma membrane. We have previously shown that the α_{1AI-II} protein can generate gating currents [1]. In this study, we demonstrate that, in addition to the ability to generate charge movements, the protein inhibits P/Q-type currents in vitro. The mechanism of inhibition of P/Q-type currents is by competition for the β subunit, as indicated by the lack of effect of the $\alpha_{1AI-IIY392S}$ protein on the current amplitude. Taken together, these data support a role for the 95kD-$\alpha_1$2.1 protein in modulating or fine-tuning neuronal responses, both by protein–protein interactions and/or modulation of P/Q-type currents.

2. Materials and methods

2.1. Antibodies

The antibodies used in this study have been previously described – Sheep 37 against the $\alpha_1$2.1/2.2 subunit [19], Sheep 49 against the β_3 subunit [10] and VD2$_1$, a monoclonal antibody, against the β subunit [8].

2.2. Enrichment of the voltage-gated calcium channels

Isolated brain regions from the rabbit brain were solubilized twice in a buffer containing 50 mM Tris, pH 7.4, 1% digitonin, 0.5 M NaCl and protease inhibitors. Equal amounts of protein were then used to enrich for the 95kD-$\alpha_1$2.1 using a combination of heparin enrichment and an antibody column to the β subunit. The extracted material was immunoblotted for the 95kD-$\alpha_1$2.1 using the Sheep 37 antibody. Aliquots of the solubilized material (100 μg of protein) were immunoblotted to examine the expression of the β_3 and β_4 subunits.

The $\alpha_1$2.1 null mice (CACNA1A−/−) have been previously described [21]. Brains were collected from 2 week old CACNA1A null and control wild-type mice. Brain microsomes were solubilized in a buffer containing 50 mM Tris, pH 7.4, 1% digitonin, 0.5 M NaCl and seven protease inhibitors and the calcium channels enriched as described above. The enriched material was immunoblotted with antibodies to the α_1 subunit (Sheep 37). The blots were then reprobed with an antibody to the β_3 subunit (Sheep 49).

2.3. Cell culture

tsA201 [22] and *mdg* (muscular dysgenic) cells [1] and baby hamster kidney (BHK) cells stably expressing the $\alpha_1$2.1/β_{1a}/$\alpha_2\delta$ subunits [12] were grown and maintained in culture as described previously.

2.4. cDNA constructs

EGFP$\alpha_1$2.1: This construct was generated by introducing an EGFP fusion protein in frame at the amino terminus of the rabbit $\alpha_1$2.1 (GenBank: X57477).

α_{1AI-II}: This construct has been previously described in [1]. The final construct contains the coding region of the $\alpha_1$2.1 up to nt 3948.

$\alpha_{1AI-IIY392S}$: An α_{1AI-II} construct with a single mutation at amino acid 392 (Y392S) was engineered from the rabbit full-length $\alpha_1$2.1Y392S. The resulting construct was similar to the α_{1AI-II} construct, except for the single amino acid change (Y to S) at position 392.

EGFPα_{1AI-II}: The EGFPα_{1AI-II} construct was generated using standard techniques. The resulting construct would encode an EGFP fusion protein of the α_{1AI-II} with the EGFP at the amino terminus of the α_{1AI-II}.

EFGP$\alpha_{1AI-IIY392S}$: This construct was derived by replacing a fragment of the EGFPα_{1AI-II} with one from the $\alpha_{1AI-IIY392S}$. The resulting construct encodes a protein similar to the EGFPα_{1AI-II}, except for a single amino acid change (Y to S) at position 392.

β subunits: The rat β_3 and β_4 subunits in the expression vector pCDNA3 and the β_{1b} and β_{2a} subunits in the pSG5 expression vector were used in this study.

2.5. Transient transfection

All transfections for expression, co-immunoprecipitation and microscopy were carried out using the Ca^{2+}-phosphate technique that has been previously described [22]. Transfection in *mdg* myotubes was performed with the polyamine LT-1 (Panvera, Madison, WI, USA) by mixing the cDNA of interest with CD8 cDNA encoding vector. Transfected cells were identified by expression of the CD8 antigen verified through binding to anti-CD8 beads. In case of the stable BHK cell line expressing the $\alpha_1$2.1/β_{1a}/$\alpha_2\delta$ subunits, transient transfections were performed using the Ca^{2+}-phosphate method or the Lipofectamine Plus Reagent (Gibco BRL, Grand Island, NY, USA). In order to select positively transfected cells for electrophysiological recording, a plasmid cDNA encoding the green fluorescent protein (GFP; pGreen Lantern-1, Gibco BRL) was also added to the DNA transfection mixture (molar ratio 1:1). Additionally, cells were transfected with GFP only as a control for non-specific effects of overexpression.

2.6. Cell lysate preparation

TsA201 cells were transiently transfected as described above. Cells were washed in phosphate-buffered saline (PBS) and then lysed in 1 ml of buffer containing 50 mM HEPES, pH 7.4, 0.4 M NaCl, 1% Triton X and 1 mM EDTA and protease inhibitors. The solubilized material was isolated by centrifugation at 15 000 rpm for 15 min at 4°C.

2.7. Co-immunoprecipitation

Cell lysates from transiently transfected cells were used for immunoprecipitation using an antibody to the β subunit. The immunoprecipitated material was separated on a gel and immunoblotted with affinity-purified antibodies (Sheep 37). Aliquots of the cell lysate were also immunoblotted.

2.8. Confocal microscopy

TsA201 cells were transfected as described above with the cDNA constructs encoding the EGFPα_{1AI-II} or the EGFP$\alpha_{1AI-IIY392S}$ with or without the β subunit. Approximately 48 h after transfection, the cells were washed and replated on coverslips. They were further incubated for approximately 24 h. The cells were washed in PBS and fixed in 4% paraformaldehyde. After extensive washing, the coverslips were mounted in Permafluor (Shandon, PA, USA). In some cases, the cells were permeabilized and treated with propidium iodide to visualize the cell nuclei. All images were obtained on a confocal microscope (Bio-Rad, CA, USA) with a magnification of 200×. More than 200 cells from independent transfections were counted for each set. Student's *t*-test was used for data analysis and $P < 0.05$ was used as significant. The data were plotted using Sigma Plot and results are presented as mean ± S.E.

2.9. Electrophysiology

Ba^{2+} currents through Ca^{2+} channels were recorded from BHK cells according to the whole-cell patch-clamp technique. All recordings were performed at room temperature using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA, USA) and 2–4 MΩ micropipettes manufactured from borosilicate glass capillary tubes. To avoid problems with spatial control of currents, only individual cells were selected for recording. Unless stated, cells were clamped at a holding potential of −90 mV and capacity transients were electronically compensated. Currents were evoked by 40-ms depolarizing voltage steps (0.05 Hz) to test potentials ranging from −40 to +70 mV. Linear leak and residual capacity currents were subtracted on-line using a P/4 standard protocol. Current records were captured on-line and digitized at a sampling rate of 20 kHz following filtering of the current record (5 kHz; four-pole Bessel filter) using a personal computer attached to a TL-1 interface (Axon). Pulse protocols, data capture and analysis of recordings were performed using pCLAMP software (Axon). Cells were bathed in a solution containing (in mM): $BaCl_2$ 10; tetraethylammonium chloride (TEA-Cl) 125; HEPES 10; glucose 5. Bath solution was adjusted to pH 7.3 with TEA-OH and 300 mosml l^{-1} with sucrose. The internal (patch pipette) solution consisted of (mM): CsCl 135; $MgCl_2$ 5; EGTA 10; HEPES 10; ATP (magnesium salt) 4; GTP 0.1 (pH 7.3/CsOH, 286 mosml l^{-1}).

3. Results

3.1. The 95kD-$\alpha_1$2.1 protein of the voltage-gated P/Q-type calcium channel is enriched in the cerebellum

We have previously described the existence of the two-domain 95kD-$\alpha_1$2.1 in the rabbit brain [19]. To determine the distribution of the protein in different regions of the brain,

which would be related to its functional roles, we decided to examine the expression of the 95kD-$\alpha_1$2.1 in the cerebellum, cerebral cortex, hippocampus and thalamus from the rabbit brain. The 95kD-$\alpha_1$2.1 was enriched using a technique similar to the one that has been previously described [19]. The enriched material was immunoblotted to examine the presence of the 95kD-$\alpha_1$2.1 protein. Aliquots of the solubilized material were also immunoblotted with different antibodies to examine the expression of the other subunits of the voltage-gated calcium channels (Fig. 1). Our results clearly demonstrate that the 95kD-$\alpha_1$2.1 protein is enriched in the cerebellum and, to a lower extent, in the cerebral cortex. Expression is also observed in the hippocampus and the thalamus. The expression levels of the β_4 subunit in the cerebellum and cortex closely appear to follow that of the 95kD-$\alpha_1$2.1. On the other hand, the β_3 subunit is enriched in the cerebral cortex and thalamus and is expressed at a lower level in the hippocampus. The β_3 is also expressed in the cerebellum, but to a much lower extent.

3.2. *The 95kD-$\alpha_1$2.1 protein of the voltage-gated P/Q-type calcium channel is a product of the CACNA1A gene*

To determine whether the 95kD-$\alpha_1$2.1 protein of the voltage-gated P/Q-type Ca^{2+} channels is a product of the CACNA1A gene, we examined the presence of the protein in a mouse with a targeted deletion of the CACNA1A gene that completely eliminates both the transcript and the $\alpha_1$2.1 protein [21]. We enriched the channels from the brains of the CACNA1A null mice and age-matched wild-type control mice using a heparin column followed by immunoaffinity chromatography. The enriched material was then immunoblotted with an antibody that recognizes the 95kD-$\alpha_1$2.1 (Fig. 2). The blots were then reprobed with an antibody to the β subunit to demonstrate the enrichment of the β_3 subunit. Our data show that though the β_3 subunit is enriched in the preparations, the 95kD-$\alpha_1$2.1 protein is absent in the CACNA1A null mice, confirming that both the full-length $\alpha_1$2.1 and the two-domain 95kD-$\alpha_1$2.1 are products of the same gene.

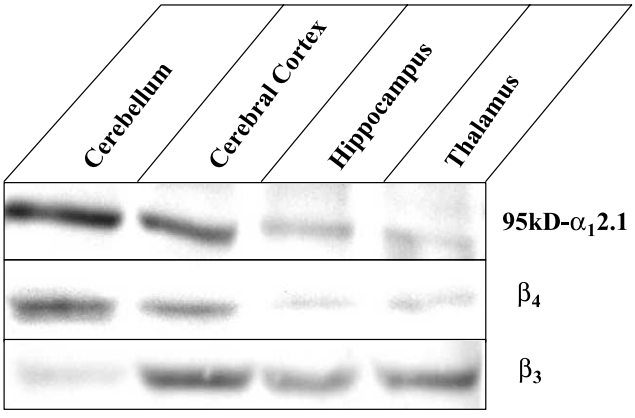

Fig. 1. The 95kD-$\alpha_1$2.1 protein is enriched in the cerebellum. The cerebellum, cerebral cortex, hippocampus and thalamus from the rabbit brain were homogenized and the voltage-gated calcium channels enriched using heparin chromatography and an antibody column to the β subunit. The presence of the 95kD-$\alpha_1$2.1 was examined using an antibody to the $\alpha_1$2.1/2.2 subunit (Sheep 37). The 95kD-$\alpha_1$2.1 is enriched in the cerebellum and the cerebral cortex with lower expression in the hippocampus and thalamus.

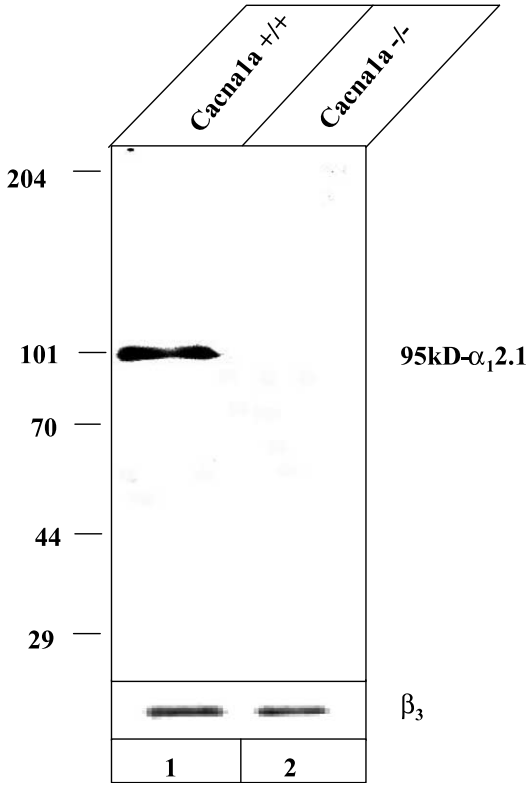

Fig. 2. The 95kD-$\alpha_1$2.1 protein is a product of the CACNA1A gene. Brain microsomes from wild-type (+/+) and CACNA1A null (−/−) mice were homogenized and the voltage-gated Ca^{2+} channels enriched using heparin affinity chromatography and enrichment with an antibody to the β subunit. The presence of the 95kD-$\alpha_1$2.1 protein was examined in the immunoprecipitated material by immunoblotting with an antibody to the $\alpha_1$2.1/2.2 subunit (Sheep 37). The 95kD-$\alpha_1$2.1 protein is absent in the CACNA1A null mice. The blot was then reprobed with an antibody to the β_3 subunit to demonstrate enrichment of the channels.

3.3. *Expression of recombinant two-domain constructs in tsA201 cells*

To assess the auxiliary subunit interactions and examine the ability of the two-domain form of the $\alpha_1$2.1 subunit to conduct voltage-gated calcium currents, we engineered a construct that extends from the N terminal of the $\alpha_1$2.1, through the first two sets of transmembrane domains and part of the II–III loop (Fig. 3A). From available data, this would be a good approximation of the native protein [19]. A similar construct with a mutation (Y392S) in the alpha interaction domain (AID) was also engineered (Fig. 3A) to study interaction of the protein with the β subunit. The AID [23] is a high-affinity interaction site that mediates its interaction with a corresponding region on the β subunit [24]. A single mutation (Y392S) in this site has been previously shown to inhibit the interaction of the α_1 and the β subunits [23]. Similar two-domain constructs with enhanced green fluorescent protein (EGFP) fusion proteins at the N terminus (Fig. 3A) were also generated to allow studies on the trafficking of the protein. Previous studies have shown that the presence of an EGFP tag at the amino terminus of the $\alpha_1$2.1 subunit does not alter its electrophysiological properties [25]; hence we chose to insert the EGFP at the N terminus of the protein.

The expression of the constructs was examined by transient

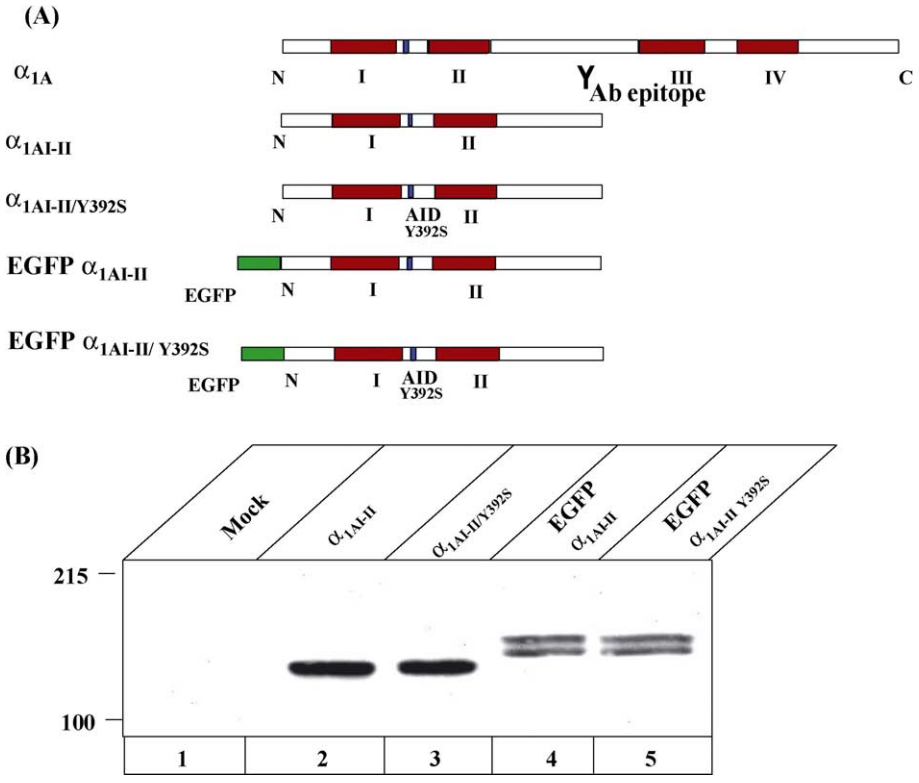

Fig. 3. Schematic of constructs and expression in mammalian cells. A: Four different constructs were used in this study. The α_{1AI-II} contains the portion of the rabbit $\alpha_1$2.1 subunit from the N-terminal and the first two sets of transmembrane domains through a part of the II–III loop. The $\alpha_{1AI-IIY392S}$ is similar to the α_{1AI-II}, except for a single point mutation, Y392S, in the AID region containing the high-affinity binding site for the β subunit. The EGFPα_{1AI-II} and EGFP$\alpha_{1AI-IIY392S}$ constructs are similar to the α_{1AI-II} and $\alpha_{1AI-IIY392S}$, respectively, except that they encode an EGFP fusion at the N terminus of the protein. The schematic of the full-length $\alpha_1$2.1/α_{1A} with the four transmembrane domains is shown for comparison. The antibody recognition epitope (Y) in the II–III loop is indicated. B: Heterologous expression of constructs in mammalian cells. tsA201 cells, transiently transfected with the constructs described above, were lysed and the expression of the proteins was examined by immunoblotting with an antibody to the α_1 subunit. All the constructs showed expression of the proteins.

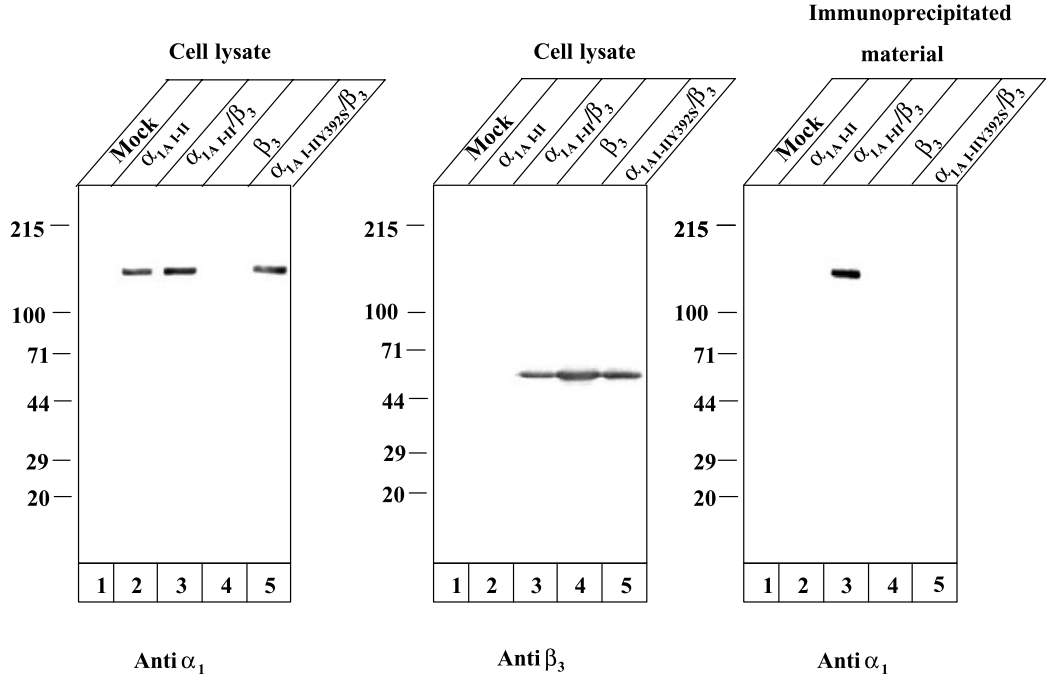

Fig. 4. The α_{1AI-II} and the β_3 subunits can form a complex. tsA201 cells were transiently transfected with either no DNA, α_{1AI-II}, α_{1AI-II} and β_3, β_3 only or $\alpha_{1AI-IIY392S}$ and β_3. The cells were lysed and immunoprecipitated with an antibody to the β subunit. The immunoprecipitated material was immunoblotted to detect the presence of the α_{1AI-II} and the $\alpha_{1AI-IIY392S}$. The cell lysates were also immunoblotted to examine the expression of the indicated proteins. The antibodies used are indicated.

transfection in tsA201 cells and immunoblot analysis (Fig. 3B). No endogenous protein was detected in the cells by the antibody, consistent with the lack of endogenous channel expression. All the constructs directed robust expression of the corresponding proteins in the transfected cells. Intriguingly, the signal in the cells transfected with the EGFP-tagged constructs was observed as doublets. Although the identity of such doublets is unknown, we speculate that these represent proteolytic degradation during cell lysis.

3.4. The α_{1AI-II} forms a complex with the β subunit

Since the α_{1AI-II} protein contains the AID, we speculated that it might form a complex with the β subunit. This interaction was examined in a cell culture system. To this end, tsA201 cells were transiently transfected with different combinations of recombinant subunits. Lysates from these cells were then used to immunoprecipitate the β subunit and the proteins that associate with it. The immunoprecipitates were examined for the presence of the α_{1AI-II} and $\alpha_{1AI-IIY392S}$ proteins (Fig. 4). Aliquots of the cell lysate were also immunoblotted to confirm expression of the transfected constructs. Consistent with the lack of endogenous β subunits immunoprecipitated by the antibody, the α_{1AI-II} does not immunoprecipitate in the absence of transiently expressed β subunit. Conversely, in the absence of transfected α_{1AI-II}, there is no endogenous protein detected by the α_1 antibody associated with the β subunit. When both the α_{1AI-II} and the β_3 are co-expressed, they can be immunoprecipitated, confirming the ability of the α_{1AI-II} protein to form a stable complex with the β_3 subunit. Further, a mutation in the high-affinity AID motif ($\alpha_{1AI-IIY392S}$) severely inhibits the interaction, confirming that the AID is indeed the predominant site of the interaction between the α_1 and the β subunits.

3.5. EGFPα_{1AI-II} is trafficked to the plasma membrane in the presence of the β subunits

To examine the ability of the α_{1AI-II} to translocate to the plasma membrane, we took advantage of the EGFP-tagged constructs. tsA201 cells were transiently transfected with these constructs in the presence or absence of the β subunits and the localization of the EGFP-tagged proteins was examined using confocal microscopy. For every set of transfections, trans-

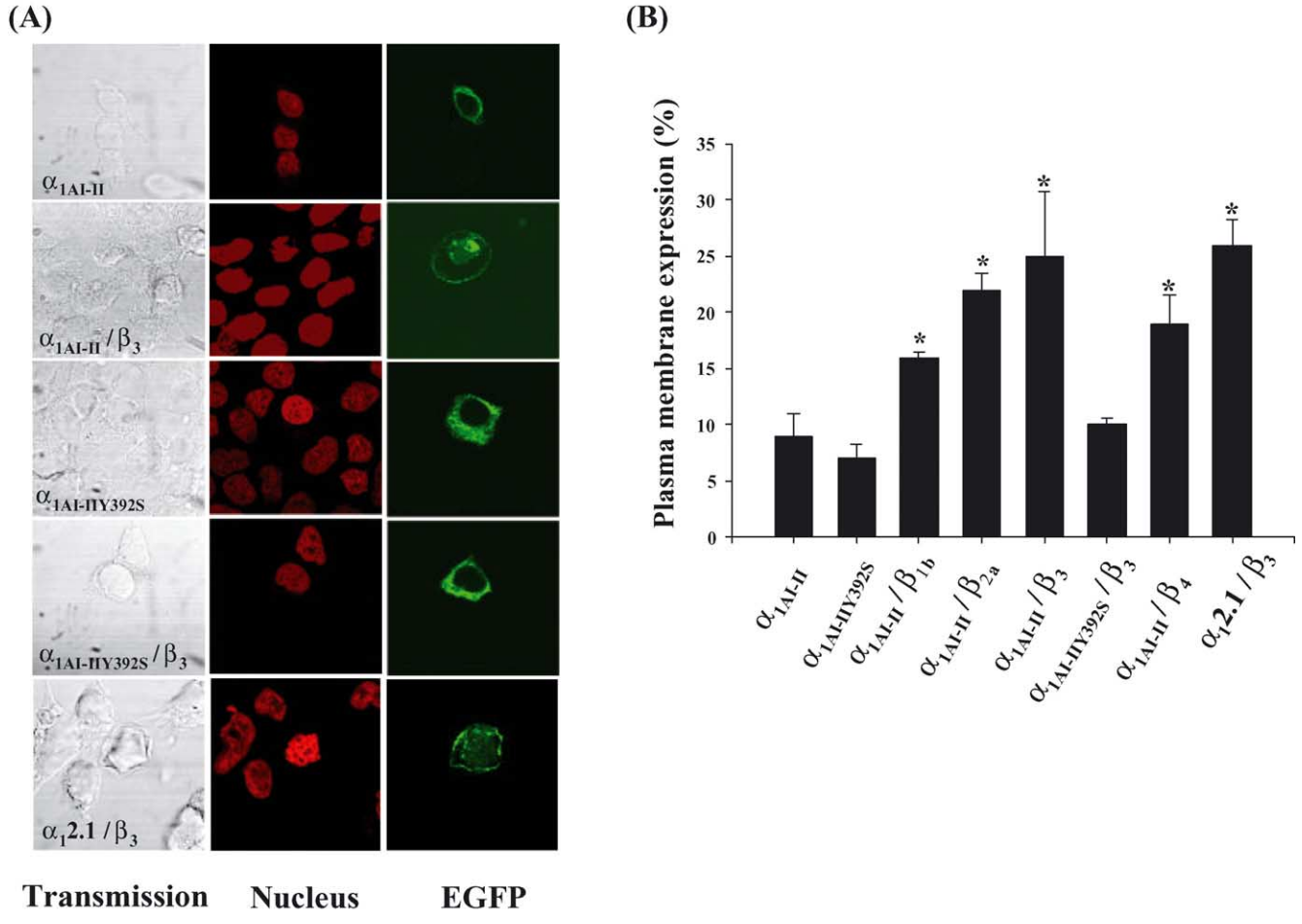

Fig. 5. Trafficking of the EGFPα_{1AI-II} to the plasma membrane in the presence of the β subunit. A: tsA201 cells were transiently transfected with (a) EGFPα_{1AI-II}, (b) EGFPα_{1AI-II} and β_3, (c) EGFP$\alpha_{1AI-IIY392S}$, (d) EGFP$\alpha_{1AI-IIY392S}$ and β_3, (e) EGFP$\alpha_1$2.1 and β_3. The cells were fixed and the localization of the EGFP-tagged proteins was examined by confocal microscopy. Shown are transmission images, the nucleus as visualized by propidium iodide staining and fluorescence images from the EGFP-tagged constructs. B: Cells from independent transfections of the EGFPα_{1AI-II}, EGFP$\alpha_{1AI-IIY392S}$ and EGFP$\alpha_1$2.1 in the presence or absence of the β subunits were counted to estimate the number of cells that demonstrated plasma membrane or internal membrane staining. The plot shows the percentage of cells for each set of transfections that exhibited membrane staining ($^*P < 0.05$). Bars represent standard errors.

fected cells were randomly counted and the numbers of cells demonstrating plasma membrane or internal staining were estimated. Statistical analysis was carried out on the numbers obtained from different transfections. The EGFPα_{1AI-II} and the EGFP$\alpha_{1AI-IIY392S}$ proteins were predominantly localized in internal membranes (Fig. 5A). Co-expression of the β_3 subunit allowed a significant (Fig. 5A,B) increase in the plasma membrane localization of the EGFPα_{1AI-II} protein (Fig. 5A). A similar effect was observed upon co-transfection with any of the other β subunits, namely β_{1b}, β_{2a} and β_4 (Fig. 5B). Consistent with the severe disruption of the α_{1AI-II}/β_3 interaction by the presence of the AID mutation, the EGFP$\alpha_{1AI-IIY392S}$ did not show this enhanced trafficking in the presence of the β_3 subunit (Fig. 5A,B). We have previously demonstrated that the two-domain α_{1AI-II} protein, in association with the β subunit, can generate gating currents [1]. This is consistent with the ability of the α_{1AI-II} protein to traffic to the plasma membrane in association with the β subunit. To compare these results with those for the full-length $\alpha_1 2.1$ subunit, we examined the membrane localization of an EGFP tagged $\alpha_1 2.1$ subunit, co-expressed with the β_3 subunit. The $\alpha_1 1.1$ protein was localized to the membrane (Fig. 5A) to a similar extent as the α_{1AI-II} protein (Fig. 5B). These results confirm both the ability of the EGFPα_{1AI-II} to be localized at the plasma membrane and the importance of the association with the β subunit for the localization, similar to that observed with the $\alpha_1 2.1$.

3.6. The α_{1AI-II} protein reduces the current amplitude of $\alpha_1 2.1/\beta_{1a}/\alpha_2\delta$-1 channels

To examine the effect of the two-domain α_{1AI-II} protein on currents generated by the full-length $\alpha_1 2.1$ subunit, the α_{1AI-II} protein was transfected in a cell line (BHK) stably expressing the $\alpha_1 2.1/\beta_{1a}/\alpha_2\delta$-1 subunits [12]. To allow identification for electrophysiological recording, cells were also co-transfected with a cDNA encoding the GFP. In addition, as a control for non-specific effects of overexpression of recombinant proteins, cells were transfected with the GFP construct alone. Voltage-gated Ca^{2+} currents were then recorded using the whole-cell patch-clamp technique. Co-expression of the α_{1AI-II} protein significantly reduced the current amplitude generated by the $\alpha_1 2.1/\beta_{1a}/\alpha_2\delta$-1 channels (Fig. 6A). Hence, total whole-cell current amplitudes at maximally activating voltages decreased about 30%, from a control value of -1794 ± 124 pA ($n = 9$) in untransfected BHK cells to -1263 ± 232 pA ($n = 10$) in the α_{1AI-II}-expressing cells ($P < 0.05$). However, the kinetic properties of the currents, including voltage dependence, activation and inactivation, remained unaltered (data not shown). Consistent with the specificity of this effect, peak current density in BHK cells transfected with GFP alone (-1776 ± 278 pA, $n = 10$) was virtually the same as in the control cells. These data show that the α_{1AI-II} protein significantly reduces the current amplitude of the $\alpha_1 2.1/\beta_{1a}/\alpha_2\delta$-1 channels. One possible explanation for the inhibition of $\alpha_1 2.1$ currents is that α_{1AI-II} could act to sequester free β subunits, and therefore limit the amount of $\alpha_1 2.1$ trafficked to the plasma membrane. To test this, BHK cells were transfected with the α_{1AI-II} construct with a point mutation (Y392S). As shown in Fig. 6A,B no inhibitory effect was observed after transfection of $\alpha_{1AI-IIY392S}$ in cells expressing the full-length $\alpha_1 2.1$ subunit. I_{Ba} current amplitude at +20 mV in cells expressing $\alpha_1 2.1$ together with the $\alpha_{1AI-IIY392S}$ construct was -641 ± 117 pA ($n = 13$), a non-significant change compared with -578 ± 62 pA ($n = 10$) for $\alpha_1 2.1$ in the absence of $\alpha_{1AI-IIY392S}$ recorded in parallel from the same transfections ($P > 0.05$). The differences in basal current amplitudes in the cells used as controls for the α_{1AI-II} and the $\alpha_{1AI-IIY392S}$ are presumably due to the different passage numbers of the cell lines used for these recordings. Taken together, the results of the electrophysiological experiments suggest that β subunit scavenging is a likely mechanism underlying the inhibitory effect of α_{1AI-II}.

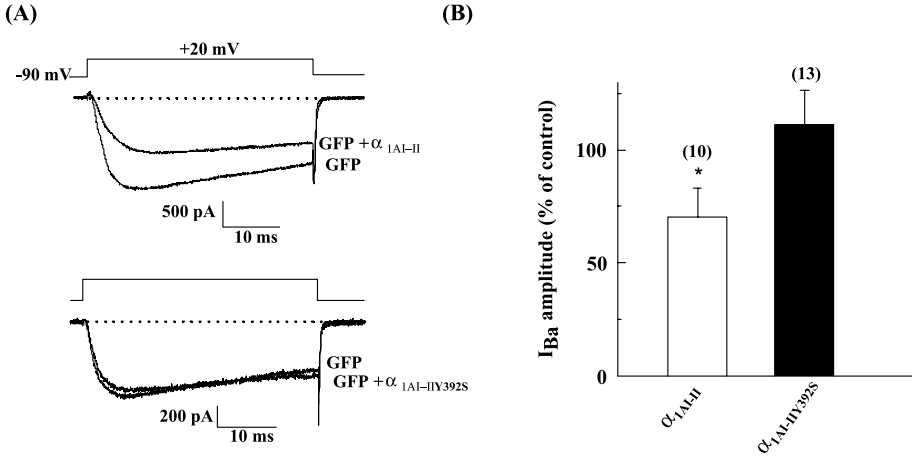

Fig. 6. Current-amplitude reduction of the $\alpha_1 2.1/\beta 1_a/\alpha_2\delta$-1 channels with the α_{1AI-II} protein. A: Representative traces of Ba^{2+} currents induced by activating pulses in BHK cells stably expressing $\alpha_1 2.1/\beta 1_a/\alpha_2\delta$-1 channels and transiently transfected with two α_{1AI-II} cDNA constructs in the presence of GFP. Upper traces correspond to the currents recorded in control GFP only transfected cells and in BHK cells transfected with the α_{1AI-II}. Lower current records were obtained in cells expressing GFP alone or GFP plus $\alpha_{1AI-IIY392S}$. B: Comparison of peak Ba^{2+} current amplitude in response to an activating pulse to +20 mV from a holding potential of −90 mV. Data are given as mean ± S.E., and the number of recorded cells is indicated in parentheses. Statistical significance of the difference between α_{1AI-II}-transfected and control cells was determined by Student's *t*-test (*$P < 0.05$).

4. Discussion

The 95kD-$\alpha_1$2.1 protein [19] is a two-domain form of the four-domain $\alpha_1$2.1 Ca^{2+} channel subunit, extending from the amino terminal of the full-length protein through a part of the II–III loop. In the present study we show that: (i) the 95kD-$\alpha_1$2.1 protein is enriched in the cerebellum; (ii) the 95kD-$\alpha_1$2.1 protein is a product of the CACNA1A gene; and (iii) a similar recombinant protein can form a complex with the β subunit, translocate to the plasma membrane and function both as a voltage sensor [1] and an inhibitor of P/Q-type Ca^{2+} currents.

Our data clearly demonstrate that the 95kD-$\alpha_1$2.1 is enriched in the cerebellum and is expressed in the cerebral cortex, hippocampus and thalamus. Interestingly, the $\alpha_1$2.1 is also expressed in these regions [21,26]. It would be interesting to assess if the generation of the 95kD-$\alpha_1$2.1 and $\alpha_1$2.1 proteins is regulated in parallel. The β_4 subunit is also robustly expressed in the cerebellum and the cortex. It has been previously shown that the β_4 subunit is the predominant subunit associated with the $\alpha_1$2.1 subunit. It is tempting to speculate that the β_4 subunit might then also be the predominant subunit associated with the 95kD-$\alpha_1$2.1 in vivo. However, further studies are required to assess this association.

It is established that the α_1 subunits are capable of interacting with any of the four known β subunits and that the AID is the predominant mediator of this interaction. This can give rise to heterogeneity in the composition of the channel complexes [10,27], hence contributing to the diversity of the channels in terms of biophysical characteristics and function. We demonstrate that the α_{1AI-II} interacts with the β_3 subunit, predominantly via the AID. This implies that all the β subunits interact with the 95kD-$\alpha_1$2.1 protein, thus resulting in heterogeneous complexes and functional diversity.

The four-transmembrane domain structure is common to the pore-forming subunit of the voltage-gated channel superfamily. Alternatively spliced transcripts that encode truncated forms of these subunits have been reported for a number of channels [28–31]. Most of these have been considered as evolutionary precursors of the ion-conducting subunits of the voltage-gated channel superfamily with no known function. It is interesting that the α_{1AI-II} does not conduct voltage-gated currents, similar to what has been observed for other two-domain proteins. Our results suggest the two-domain form of the 95kD-$\alpha_1$2.1 subunit may have specialized physiological roles, raising the possibility that the other truncated channel proteins may represent evolutionary precursors with significant physiological function.

The exact nature of physiological origin of the 95kD-$\alpha_1$2.1 protein is unclear. The mechanism of generation of the other two-domain proteins suggests that an alternative transcript generates the 95kD-$\alpha_1$2.1 protein. We have previously demonstrated [19] that the 95kD-$\alpha_1$2.1 protein is similar to the $\alpha_1$2.1 in terms of the amino terminal sequence, suggesting a common transcriptional start site for both proteins. Interestingly, a variant of the $\alpha_1$2.2 arises from an alternative transcript in which the known exon–intron boundaries are not retained [32]. The mechanism of generation of this transcript is also unclear, suggesting that alternative transcript-generating mechanisms may exist.

Our data demonstrate that the EGFPα_{1AI-II} can localize to the plasma membrane. As with the $\alpha_1$2.1 subunit, this membrane trafficking is augmented by the co-expression of the β subunit. The role of the β subunits in the membrane trafficking of the α_1 subunits has been well documented. Recent data suggest that this may be due to the ability of the β subunit to circumvent the endoplasmic reticulum retention signal in the α_1 subunits [33]. Presumably, similar mechanisms operate in the trafficking of the 95kD-$\alpha_1$2.1 and the α_{1AI-II} to the plasma membrane. Moreover, our data suggest an important role for the first half of the $\alpha_1$2.1 in the trafficking of the protein to the plasma membrane. The ability of the AID mutation (Y392S) to restrict the membrane trafficking of the α_{1AI-II} protein is also consistent with previous studies that demonstrate the importance of this site in the association of the α_1 and β subunits, and also the significance of this interaction for the ability of the β subunit to aid the trafficking of the protein to the membrane [34].

We have previously demonstrated that the truncated two-domain α_{1AI-II} protein, in association with the β subunit, can generate gating currents in dysgenic myotubes [1]. The ability of the α_{1AI-II} to function as a voltage sensor, similar to that observed with a two-domain form of the $\alpha_1$1.1 [1], indicates that the protein can undergo conformational changes in response to membrane depolarization. The P/Q-type channels are coupled to the exocytotic machinery and serve to link the depolarization-dependent Ca^{2+} influx to neurotransmitter release. The II–III loop of the $\alpha_1$2.1 subunit serves as a binding site for a number of proteins associated with the exocytotic machinery including syntaxin, SNAP-25 [35], synaptotagmin [36] and cysteine string proteins [37]. Since the 95kD-$\alpha_1$2.1 protein may possess at least some of the sites required for interaction with these proteins, we anticipate that it may interact with the synaptic vesicle proteins and couple depolarization to Ca^{2+}-insensitive neurotransmitter release [38,39] by conformational changes associated with the movement of gating charges.

Alternatively, the 95kD-$\alpha_1$2.1 protein may couple to the ryanodine receptor in the brain and be involved in intracellular Ca^{2+} release, via conformational coupling, similar to that observed with Ca^{2+}-independent excitation–contraction coupling in the skeletal muscle. The association of the neuronal voltage-gated calcium channels with the ryanodine receptor is only beginning to be revealed [40] and there is currently no evidence to indicate that the non-L-type voltage-gated channels couple to the ryanodine receptor in the brain. However, given the similarity between the different α_1 subunits and the wide expression of the ryanodine receptor in the brain, this interaction may be possible in vivo. We propose that the 95kD-$\alpha_1$2.1 protein may be involved in coupling to either of the ryanodine receptor isoforms in the brain to mediate activation of the receptor, and hence calcium release and intracellular calcium signaling, by mechanical coupling in response to membrane depolarization.

In neurons, the inhibitory function of the 95kD-$\alpha_1$2.1 protein might serve as a mechanism for regulation of current amplitude of the P/Q-type channels and hence neurotransmitter release and synaptic activity. As a precedent for this kind of regulatory role in voltage-gated channels, it has been demonstrated that truncated forms of the potassium channel KvLQT1 [41] and truncated forms of the ascidian L-type Ca^{2+} channel α_1 subunit [42] can effectively reduce the current amplitude of the full-length subunits. The α_{1AI-II} protein reduces the current amplitude of the $\alpha_1$2.1 subunit by direct

competition for the β subunit. Interestingly, it has been demonstrated that a truncated form of the $\alpha_1$2.2 protein, similar in structure to the 95kD-$\alpha_1$2.1 protein, inhibits the currents generated by the full-length $\alpha_1$2.2 protein [43]. The mechanism for this inhibition appears to be a direct effect of the truncated protein on the full-length protein and not via competition for the β subunit. It is not clear why the mechanisms of inhibition of the truncated $\alpha_1$2.1 and $\alpha_1$2.2 proteins differ, though it is possible that this is related to the intrinsic nature of the two pore-forming subunits.

Finally, genetic mutations in the CACNA1A gene have been associated with episodic ataxia type 2, which is a rare autosomal dominant disorder. Most of the known mutations are predicted to result in truncated proteins [44], which retain only some of the transmembrane domains of the full-length protein. The mechanisms by which these mutations cause disease are not well understood. This study presents evidence that raises the possibility that the truncated proteins produced in the pathological conditions may possess physiological function and result in the disease phenotype by a gain of function or inappropriate dominant inhibition of the P/Q-type channel activity in the nervous system.

In summary, our data support a physiological role for the 95kD-$\alpha_1$2.1 protein, in association with the β subunit, as a voltage sensor and in the regulation of P/Q-type Ca^{2+} currents. Further studies are necessary to delineate the cells and systems in which these roles are important and to determine whether the protein arises by alternate splicing, editing or post-translational modification of the full-length $\alpha_1$2.1 protein. It would also be interesting to examine if the full-length and truncated forms of the protein are expressed in the same or different cell types and whether they are differentially regulated by extracellular stimuli or during development. Such studies might reveal important roles for the protein in critical neuronal processes including synaptic transmission and plasticity.

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