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Immunophilin Deficiency Augments Ca^{2+} -Dependent Glutamate Release from Mouse Cortical Astrocytes

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SUMMARY

Immunophilins are receptors for immunosuppressive drugs such as the macrolides cyclosporin A (CsA) and FK506; correspondingly these immunophilins are referred as cyclophilins and FK506-binding proteins (FKBPs). In particular, CsA targets cyclophilin D (CypD), which can modulate mitochondrial Ca^{2+} dynamics. Since mitochondria have been implicated in the regulation of astrocytic cytosolic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) dynamics and consequential Ca^{2+} -dependent exocytotic release of glutamate, we investigated the role of CypD in this process. Cortical astrocytes isolated from CypD deficient mice *Ppif*^{-/-} displayed reduced mechanically-induced $\text{Ca}^{2+}_{\text{cyt}}$ increases, even though these cells showed augmented exocytotic release of glutamate, when compared to responses obtained from astrocytes isolated from wild-type mice. Furthermore, acute treatment with CsA to inhibit CypD modulation of mitochondrial Ca^{2+} buffering, or with FK506 to inhibit FKBP12 interaction with inositol-trisphosphate receptor of the endoplasmic reticulum, led to similar reductive effects on astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ dynamics, but also to an enhanced Ca^{2+} -dependent exocytotic release of glutamate in wild-type astrocytes. These findings point to a possible role of immunophilin signal transduction pathways in astrocytic modulation of neuronal activity at the tripartite synapse.

INTRODUCTION

At the tripartite synapse [1], bidirectional astrocyte-neuron signaling can occur via Ca^{2+} -dependent vesicular release of glutamate [2–4]. The source of cytosolic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) for such release of glutamate from astrocytes is dual: (i) predominately from the endoplasmic reticulum (ER) internal store involving both inositol (1,4,5)-trisphosphate (IP_3)- and ryanodine/cafeine-receptors [5]; (ii) the extracellular Ca^{2+} entry via the transient receptor potential canonical 1 (TRPC1) channel [6]. Additionally, mitochondria act as a source/sink of $\text{Ca}^{2+}_{\text{cyt}}$ necessary for exocytotic glutamate release from astrocytes [7].

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Mitochondria can sequester free Ca^{2+} through the Ca^{2+} uniporter [8,9] and subsequently release free Ca^{2+} to the cytosol via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [10]. Another regulator of mitochondrial Ca^{2+} is cyclophilin D (CypD), a peptidyl-prolyl *cis-trans* isomerase (PPIase) F, and a member of the cyclophilin family that have eight subtypes in mammals [11]. CypD is a matrix mitochondrial protein involved in modulation of the mitochondrial permeability transition pore (MPTP) affinity for Ca^{2+} . MPTP is a high conductance channel, whose stabilization in the open conformation results in an increase of mitochondrial inner membrane permeability to solutes in pathology [12,13], while transient openings of the pore may serve a physiological role as a mechanism to remove Ca^{2+} rapidly from mitochondria [14]. We previously provided evidence that the MPTP plays a role in rapid delivery of Ca^{2+} from mitochondria to cytosol for the exocytotic release of glutamate [7]. The agent CsA inhibits opening of the MPTP after binding to CypD [15], and has been shown to increase the capacity of mitochondria to uptake more Ca^{2+} in cortical astrocytes [16]. We investigated the role of CypD in astrocytic Ca^{2+} signaling and subsequent glutamate release by using CypD knock-out (KO) mouse model *Ppif*^{-/-}. We found that CypD KO astrocytes display attenuated mechanically-induced $\text{Ca}^{2+}_{\text{cyt}}$ responses, but exhibited augmented exocytotic release of glutamate. Similar effects on astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ dynamics and consequential Ca^{2+} -dependent exocytotic release of glutamate in wild-type astrocytes could be observed upon acute treatment with CsA that inhibits CypD function in mitochondrial Ca^{2+} buffering. Besides CypD, FK506-binding protein 12 (FKBP12) could be involved in modulation of astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ dynamics and Ca^{2+} -dependent exocytotic release of glutamate. Apart from the usual PPIase dependent activity of many other FKBP family proteins, FKBP12 acts as a scaffolding protein anchoring to IP_3 receptors (IP_3R) and coordinating its channel properties [17]. Indeed, we found that FKBP12 represents another signaling node for modulation of astrocytic Ca^{2+} signaling and glutamate release, since FK506 exhibited similar effects on astrocytes as CsA. It appears that the augmentation of exocytotic glutamate release by CsA/FK506 occurred downstream of Ca^{2+} likely at the level of secretory machinery. These findings implicate the involvement of immunophilin signaling pathways in exocytotic glutamate release from astrocytes and may further point to the possible utilization of such pathways in astrocyte-mediated modulation of the neuronal activity at the tripartite synapse [1] in health and disease.

MATERIALS AND METHODS

Astrocyte cultures

All animal procedures were in strict accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee. A modified culture method [7,18] was used to grow grouped and solitary astrocytes on a permissive substrate [1mg/ml polyethyleneimine (PEI); Sigma, St. Louis, MO]. Visual cortices from 1- to 4-day old C57BL/6 (genetic background/wild-type) and *Ppif*^{-/-} (a breeding pair generously provided by Dr. Michael Forte, Vollum Institute, Portland, Oregon) mice pups were dissected, treated with papain (20 I.U./ml; Sigma) diluted in Hank's Balanced Salt Solution (HBSS) (Invitrogen, Carlsbad, CA) for 1 hr at 37°C, and neutralized with trypsin inhibitor (type II-O, 10 mg/ml; Sigma) for 5 minutes at room temperature. The tissue was then washed with HBSS, immersed in culture medium containing α -MEM (without phenol red; Invitrogen) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 20 mM glucose, 2 mM L-glutamine, 1 mM sodium pyruvate, 14 mM sodium bicarbonate, penicillin (100 I.U./ml), and streptomycin (100 $\mu\text{g}/\text{ml}$) (pH 7.35), and triturated using a glass pipette. The resulting dispersion of neural cells was applied into culture flasks (25 cm^2). After 1 hour flasks were washed with HBSS and remaining attached cells at the bottom of the flasks were provided with fresh media. The cells were maintained at 37°C in a 95% air/5% CO_2 incubator for 14

to 20 days to allow for growth and proliferation to ~80% confluency. At this juncture, the cell cultures were purified for astrocytes [19]. The culture flasks were washed with cold culture medium (4°C) and then shaken on a horizontal orbital shaker (260 rpm at 37 °C) for 1.5 hours. After exchange of culture media, the flasks were shaken again for 18–19 hours. Remaining attached cells at the bottom of the flask were detached using trypsin (10,000 N ϵ -benzoyl-L-arginine ethyl ester hydrochloride units/ml; Invitrogen, Carlsbad, CA) diluted in HBSS for 2 minutes. The enzymatic reaction was terminated with the addition of fresh culture media. Cells were pelleted with centrifugation (100 $\times$ g) for 10 minutes, and then resuspended in culture medium. Cells were plated on round glass coverslips (12 mm in diameter) pre-coated with PEI and maintained in the 95% air/5% CO₂ incubator at 37°C until used in experiments after 7–9 days. This culturing method yields purified astrocytes (>99%), as we described elsewhere [6,20].

Isolation of mitochondria

Mitochondria were isolated from astrocytes plated and purified in flasks (75 cm²). After purification astrocytes were returned to the 95% air/5% CO₂ incubator at 37°C for 1 day at which point there were washed with ice cold phosphate buffered saline (PBS; pH= 7.6) containing (in mM) NaCl (137), KCl (2.7), KH₂PO₄ (1.47) and Na₂HPO₄ (8.1), followed by incubation with 0.25% trypsin-EDTA (Gibco) for 5 minutes at 37°C to detach them from the flask. The enzymatic reaction was inhibited by addition of culture media. Cells were then pelleted and resuspended in cavitation buffer (pH 7.4) composed of (in mM): HEPES (5), MgCl₂ (3), EGTA (1) and sucrose (250). Astrocytes were disrupted by nitrogen cavitation (~1.7 MPa; 5 min) while kept on ice. The resulting cell lysate was centrifuged at 950 $\times$ g for 10 min at 4°C to remove unlysed cells, large debris and nuclei. The isolated supernatant was centrifuged at 10,000 $\times$ g for 7 min at 4°C. The resulting pellet was centrifuged twice at 10,000 $\times$ g, first for 5 min, and then after washing, again for 3 min. The resulting secondary pellet constitutes a highly enriched mitochondrial fraction. Mitochondria were resuspended in cavitation buffer supplemented with 0.1% fatty acid free bovine serum albumin, briefly pelleted at 2,000 $\times$ g and shortly kept on ice until mitochondrial Ca²⁺ uptake was assessed.

Assessment of mitochondrial Ca²⁺ uptake

The mitochondrial Ca²⁺ buffering capacity was monitored using a cuvette-based fluorometer with continuous stirring and the low affinity Ca²⁺ indicator Calcium GreenTM-5N (Invitrogen). Mitochondrial pellets were resuspended (0.5 mg/ml) in a solution composed of (in mM): KCl (125), MgCl₂ (1), K₂HPO₄ (2) and HEPES (20) (pH=7.0), supplemented with glutamate (5 mM), malate (5 mM), ADP (150 μ M), oligomycin (1.2 μ g/ml), and Calcium Green-5N (25 nM). After incubation for ~240 sec, successive additions of Ca²⁺ (10 nmol in 2 μ l aliquots) boluses/spikes at 3 minutes intervals were applied. Calcium Green-5N emission was collected at 531 nm due to its excitation at 506 nm.

Western blotting

Pellets of purified astrocytes were obtained as described above. Here, the resulting pellets were washed once in PBS and then homogenized in lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 0.1 mM phenylmethylsulfonyl fluoride, and aprotinin, leupeptin, and pepstatin, (each at 10 μ g/ml)], sonicated on ice and spun at 2,000 $\times$ g for 10 min at 4°C. Protein concentration of the supernatant was determined using the bicinchoninic acid assay method (Pierce). Supernatants from cellular lysates were diluted to a final concentration of 1 mg/ml in a 2 $\times$ reducing stop buffer [0.25 M Tris-HCl (pH 7.5), 2% SDS, 25 mM dithiothreitol, 5 mM EGTA, 5 mM EDTA, 10% glycerol, and 0.01% bromophenol blue as a tracking dye] and then incubated in a boiling water bath for 5 min. Mitochondrial suspensions recovered after fluorometry were briefly sonicated and mixed to a 1:2 ratio in 2 $\times$ reducing stop buffer prior to being incubated in a boiling water

bath for 5min. Proteins were separated by SDS-PAGE on 12% gel, each lane receiving 10 µg of protein from cellular lysates or 10 µl from recovered mitochondrial suspensions. Following transfer to nitrocellulose membranes, proteins were probed using primary antibodies against ATP synthase (1:5,000; Invitrogen), CypD [1:2,000; Calbiochem (San Diego, CA)], succinate dehydrogenase/complex II 30 kDa (1:10,000) and 70 kDa (1:5,000) subunits (both from Invitrogen), porin 31HL/porin 1 (1:2,000; Calbiochem) and tubulin (1:2,500; Sigma). After washing, blots were incubated with horseradish peroxidase-conjugated secondary antibodies and developed using peroxidase substrate chemiluminescence.

Pharmacological agents

The concentrations and pre-incubation times for cyclosporin A (CsA) (20 µM, 5 min; Alexis Biochemical, San Diego, CA), and FK506 (1µM, 20 min; AG Scientific, San Diego, CA) were adapted from literature [16,21]. These agents were applied onto astrocytes in external solution (pH 7.35) containing (in mM): NaCl (140), KCl (5), CaCl₂ (2), MgCl₂ (2), HEPES (10), and glucose (5). Prior to calcium ion imaging and assessment of glutamate release, the astrocytes were pre-incubated in the pharmacological agent at room temperature (22–25 °C) for the duration stated above, and were kept bathed in the agents during the entire subsequent experimental procedure lasting ~200 seconds.

Cellular and mitochondrial staining

Visualization and quantification of cellular and mitochondrial areas were performed using the vital stain calcein acetoxymethyl (AM) ester [5,22] and 4-(4-(diethylamino)styryl)-methylpyridinium (4-Di-2-ASP) [7,23], respectively. Astrocytes were loaded with calcein AM (1 µg/ml; Invitrogen) with the addition of 0.025% w/v pluronic acid (Invitrogen) to aid solubilization in complete culture medium, at 37°C for 10 min. After de-esterification of the calcein AM for 10 min in external solution at room temperature, the astrocytes were incubated in 4-Di-2-ASP (10 µM; Invitrogen) for 3 min at room temperature. Calcein and 4-Di-2-ASP fluorescence emissions were visualized with the standard fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filter sets (Chroma; Rockingham, VT), respectively. To obtain the pixel area occupied by the cell and mitochondria, the fluorescence signal was thresholded and number of positive pixels counted with software (Image J 1.42, NIH, Washington, D.C.) [6]. The number of positive pixels occupied by mitochondria of a single cell was divided by the number of positive pixels corresponding to the entire cell in order to obtain the relative area of the cell occupied by mitochondria.

Ca²⁺ imaging

The Ca²⁺_{cyt} level of cultured solitary astrocytes were measured using the Ca²⁺ indicator fluo-3 as described elsewhere [5,7,20,24]. Astrocytes were loaded with fluo-3 AM (10µg/ml; Invitrogen) in external solution containing pluronic acid (0.025% w/v) for 30 min at room temperature. Afterwards, the cells were incubated in fresh external solution for 30 min at room temperature to allow de-esterification of fluo-3 AM, and were then incubated with pharmacological agents. The coverslips containing astrocytes were mounted onto a recording chamber, and cells were visualized with a standard FITC filter set. Fluorescence intensities obtained from somata of indicator-loaded astrocytes were subtracted by the background fluorescence obtained from regions of coverslips containing no cells. Fluorescence data were expressed as dF/Fo (%) with the cell baseline fluorescence (Fo) representing the average of the first 5 images before mechanical stimulation while dF represents the change in fluorescence emission. At least three independent cultures, with equal number of cells within different treatment groups, were used to compare the effects of

the pharmacological agents with control, as well as to compare responses from the wild-type *versus* CypD deficient astrocytes.

Extracellular glutamate imaging

Glutamate released by solitary astrocytes was measured using the L-glutamate dehydrogenase (GDH; Sigma)-linked assay as previously described [5,7,20,25]. PEI-coated coverslips containing cultured astrocytes were mounted onto a recording chamber, and the astrocytes were incubated in the external solution with pharmacological agents. A set of images containing the cell of interest were taken in a sham run to correct for reduction of fluorescence due to photobleaching. In the experimental run, the external solution was exchanged with the fresh external solution additionally containing 1 mM of β -nicotinamide adenine dinucleotide (NAD^+ ; Sigma), 55–61 I.U./ml of GDH, and in some cases the pharmacological agent. The assay was visualized with a standard 4'6-diamidino-2-phenylindole (DAPI) filter set (Nikon). When released, glutamate is oxidized by GDH to α -ketoglutarate, while bath supplied NAD^+ is reduced to NADH, a fluorescent product when excited by UV light. Fluorescence trace from the experimental run was subtracted by that obtained during the sham run. We analyzed region of interests near solitary astrocytes since they are devoid of intracellular NADH signal, and thus record extracellular NADH fluorescence which reports on released glutamate. Fluorescence data was expressed as dF/F_0 (%) with the baseline fluorescence (F_0) being the fluorescence of the media surrounding the astrocyte before mechanical stimulation. At least three independent cultures were used to compare the effect of the pharmacological agents with control. For each experimental set, equal numbers of cells from treatment groups were tested.

GDH activity assay

To account for the possibility that the pharmacological agents may have an effect on the activity of the GDH in the media surrounding the astrocytes, an assay was performed using a spectrophotometer (Genequant Pro; Cambridge, UK) and NADH absorbance as a measure of GDH activity [5,7]. The assay solution contained normal external solution supplemented with NAD^+ (1 mM), glutamate (100 μM), and ~ 59 I.U./ml of GDH. The concentrations of pharmacological agents were identical to those used for Ca^{2+} and glutamate imaging. The NADH produced from the reaction was monitored by its absorbance at 320 nm. The assay showed that at the five minute interval, which allowed the reaction to reach a steady-state level, there was no significant difference in NADH absorbance when solution containing NAD^+ , glutamate and GDH [(control; 0.43 ± 0.03 (mean $\pm$ SEM))] was compared to that additionally containing FK506 (0.41 ± 0.03 ; Student's t-test, $p = 0.98$). We have previously reported that CsA does not affect GDH-linked assay measurements [7].

Mechanical stimulation

To elicit $\text{Ca}^{2+}_{\text{cyt}}$ increase in solitary astrocytes and subsequent release of glutamate, we employed a patch pipette to deliver mechanical stimulus [26]. This particular method allows spatio-temporal control in the application of the stimulus, without affecting the integrity of the plasma membrane [5]. The patch pipette was lowered onto solitary astrocytes with a micromanipulator (Narishige, East Meadow, NY). The contact with the plasma membrane, which represents the mechanical stimulus, was monitored by measuring the change in pipette resistance using a patch-clamp amplifier (PC-ONE; Dagan, Minneapolis, MN) that delivered -20 mV, 10 msec square pulses at 50 Hz. Pipette resistances measured 2.1– 3.7 M Ω before the contact with the astrocytes, and increased to 2.4– 5.3 M Ω during the contact with astrocytes, lasting less than 1 sec.

Image acquisition and processing

An inverted microscope (TE 300, Nikon, Melville, NY), equipped with differential interference contrast (DIC), wide-field fluorescence illumination and oil-immersion objectives, was used in all experiments. For glutamate imaging we used a 40× SFluor objective (1.3 NA; Nikon), while all other experiments were done using a 60× Plan Achromatic objective (1.4 NA; Nikon). Images were acquired using a CoolSnap-HQ cooled charge-coupled device (CCD) camera (Roper Scientific Inc., Tucson, AZ) driven by V++ imaging software. For the glutamate release analysis, the dF/F_0 of the treatment groups were ranked and normalized to allow comparisons between experimental batches and accommodate for variations in GDH concentration and culture conditions. Similar ranking of intracellular Ca^{2+} dynamics dF/F_0 's was done for consistency. All images shown in figures represent raw data with their pixel intensities within the camera's (CoolSNAP-HQ) dynamic range (0–4,095).

Statistical analysis

The increase in fluo-3 and NADH fluorescence due to mechanical stimulation was tested using paired t-tests. The effect of CypD deficiency on intracellular Ca^{2+} dynamics and exocytotic glutamate release was tested using Mann-Whitney U-test. Student's t-test was used when comparing the cellular area occupied by mitochondria, as well as when comparing the levels of normalized fluo-3 and NADH fluorescence, in some cases with additional CsA or FK506 treatments, between wild-type and CypD-deficient astrocytes. Effects of CsA or FK506 on wild-type or CypD-deficient astrocytes intracellular Ca^{2+} dynamics, and glutamate release were tested using one-way ANOVA, followed by Fisher's least significant difference (LSD) test.

RESULTS

CypD KO cortical astrocytes have normal amounts of mitochondria, which have increased capacity for Ca^{2+} buffering

Astrocytes produce glutamate *de novo* from tricarboxylic acid cycle substrates in the matrix of mitochondria [27]. Once mitochondrial glutamate reaches the cytosol it represents a limiting factor in astrocytic Ca^{2+} -dependent glutamate release [18]. Consequently, we first tested the amount of mitochondria and mitochondrial proteins in CypD KO astrocytes. We labeled astrocytic mitochondria using 4-Di-2-ASP while the cellular cytosol was disclosed by labeling astrocytes with the vital dye calcein (Fig. 1A). We used fluorescence microscopy to visualize and quantify the proportion of cellular area occupied by mitochondria. We determined the pixel area associated with the fluorescence of 4-Di-2-ASP and calcein. To quantitatively assess the relative amount of mitochondria per cell we generated ratios of 4-Di-2-ASP/calcein positive pixels (Fig. 1B). In *Ppif*^{+/+} (wild-type) astrocytes (n=23) mitochondrial stain represented 0.37 ± 0.11 of the entire cell area. There was no significant difference in mitochondrial representation in CypD KO astrocytes (n= 18; 4-Di-2-ASP/calcein positive pixels = 0.34 ± 0.11 ; Student t-test, $p=0.39$; Fig. 1B). In support of these findings, immunoblot analysis of cellular lysates from wild-type and CypD KO astrocytes did not reveal major differences in the expression levels of key mitochondrial proteins localized in the matrix (ATP synthase), inner (ATP synthase and complex II subunits) and outer (porin 31HL) membranes of the organelle (Fig. 1C). However, the matrix protein CypD, which was readily detectable in wild-type astrocytes, as expected was not found in CypD KO astrocytes. Thus, it appears that wild type and CypD KO astrocytes have similar amounts of mitochondria and their proteins, with the exception of the obvious absence of CypD in CypD KO mice.

Brain mitochondria from CypD KO mice exhibit higher Ca^{2+} capacity than brain mitochondria from wild-type mice, requiring approximately twice the amount of Ca^{2+} necessary to induce MPTP than wild-type mitochondria *in vitro* [15,28,29]. Thus, we studied whether similar increase in mitochondrial Ca^{2+} capacity occurs in mitochondria isolated from CypD KO astrocytes. Here, the successive additions of 10 nmol Ca^{2+} boluses/spikes were delivered to the cuvette loaded with suspensions containing an equal amount (based on protein content) of either mitochondria from CypD KO or wild-type astrocytes (Fig. 2). Comparison of Ca^{2+} uptake kinetics in mitochondria from wild-type and CypD KO astrocytes revealed that mitochondria from wild-type astrocytes were not able to sequester Ca^{2+} effectively after a limited number of extra-mitochondrial Ca^{2+} boluses were delivered, while mitochondria from CypD KO astrocytes were able to buffer substantially higher amounts of Ca^{2+} (Fig. 2A). To confirm equal loads of mitochondria in our fluorometric assay, we post-experimentally recovered mitochondrial suspensions from the cuvettes and immunoblotted them for the assessment of mitochondrial proteins ATP synthase and porin 31HL; their expressions were similar (Fig. 2B). However, as expected, unlike in mitochondria from wild-type astrocytes, CypD was not detected in mitochondrial preparations obtained from CypD KO astrocytes (Fig. 2B). Thus, the genetic ablation of CypD resulted in a robust increase in the Ca^{2+} buffering capacity of astrocytic mitochondria, although the cellular amount of these organelles remained unaltered.

CypD deficient cortical astrocytes exhibit decreased $\text{Ca}^{2+}_{\text{cyt}}$ responses, but augmented glutamate release

Ca^{2+} -dependent vesicular glutamate release in rat cortical astrocytes is regulated by the liberation of free Ca^{2+} from the ER to the cytosol [5], and the subsequent sequestration and release of free Ca^{2+} by mitochondria [7]. Consistent with findings in plasma membrane permeabilized astrocytes [16], our previous study showed evidence that CsA increases mitochondrial Ca^{2+} sequestration, and thereby decreases $\text{Ca}^{2+}_{\text{cyt}}$ response resulting in the reduced glutamate release in mechanically stimulated rat cortical astrocytes [7]. The development of *Ppif*^{-/-} (CypD KO) mice has provided evidence that CypD is a target for CsA and a key regulator of the mitochondrial Ca^{2+} uptake capacity [15].

To further examine the contribution of CypD to the mitochondrial Ca^{2+} buffering and its effects on the Ca^{2+} -dependent glutamate release from astrocytes, we studied the mechanically-induced increase in $\text{Ca}^{2+}_{\text{cyt}}$, and in parallel experiments, the consequential glutamate release from cultured wild-type and CypD KO mouse astrocytes. Mechanical stimulation allowed us to selectively increase $\text{Ca}^{2+}_{\text{cyt}}$ in solitary astrocytes, which reduces influences of astrocyte-astrocyte communication in the measurements [5]. This direct stimulus caused a rapid and significant increase in $\text{Ca}^{2+}_{\text{cyt}}$ of wild-type (n=65; peak $\text{dF}/\text{Fo} = 570 \pm 25\%$; paired t-test, $p < 0.01$) and CypD KO (n=65; peak $\text{dF}/\text{Fo} = 527 \pm 27\%$; paired t-test, $p < 0.01$) astrocytes (Fig. 3A). Peaks of $\text{Ca}^{2+}_{\text{cyt}}$ increases corresponded to Ca^{2+} accumulations of ~ 5.9 and $4.2 \mu\text{M}$ over baseline ($\sim 70 \text{ nM}$), respectively, as determined using calibration of fluo-3 in parallel experiments as described elsewhere [6]. We further analyzed the $\text{Ca}^{2+}_{\text{cyt}}$ response of wild-type and CypD KO astrocytes, specifically the peak and cumulative $\text{Ca}^{2+}_{\text{cyt}}$ accumulation after normalization of responses using the ranking approach, as previously described [7]. We found that CypD KO cortical astrocytes display the significant reduction in peak Ca^{2+} response when compared to that recorded from wild-type astrocytes (Mann-Whitney U-test, $p < 0.01$) (Fig. 3B); no significant differences were observed in the cumulative Ca^{2+} response (Mann-Whitney U-test, $p = 0.62$) (Fig. 3C). These data are in agreement with the increased buffering capacity of mitochondria in CypD KO astrocytes (Fig. 2).

In parallel to the assessment of $\text{Ca}^{2+}_{\text{cyt}}$ dynamics, we recorded mechanically-induced Ca^{2+} -dependent glutamate release from wild-type and CypD KO cortical astrocytes. Here, using a

region of interest near solitary astrocytes, we recorded the accumulation of NADH that reports on glutamate released from astrocytes to the extracellular space [5]. Mechanical stimulation caused a reliable increase in the NADH fluorescence in the vicinity of wild-type ($n=42$; peak NADH $dF/F_0 = 40 \pm 4\%$; paired t-test, $p<0.01$) and CypD KO ($n=42$; peak NADH $dF/F_0 = 56 \pm 10\%$; paired t-test, $p<0.01$) astrocytes (Fig. 3D). The peak fluorescence increases corresponded to extracellular glutamate accumulation of ~ 0.8 and $1.2\ \mu\text{M}$, respectively, when using the calibration of GDH-linked assay, as described elsewhere [25]. Further detailed analysis of NADH accumulations after normalization indicated that CypD KO astrocytes exhibited significant increase in both the peak (Mann-Whitney U-test, $p<0.02$; Fig. 3E) and cumulative (Mann-Whitney U-test, $p<0.01$; Fig. 3F) extracellular glutamate release due to mechanical stimulation. Thus, genetic ablation of CypD results in decreased astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ response due to mechanical stimulation, but with consequential glutamate release augmented. However, it should be noted, that Ca^{2+} -dependent glutamate release is proportional to $\text{Ca}^{2+}_{\text{cyt}}$ response [30] where the increase in $\text{Ca}^{2+}_{\text{cyt}}$ is sufficient and necessary for this release to occur [24]. Taken together, these data may implicate a possible modulation of Ca^{2+} -dependent glutamate release from astrocytes down-stream of $\text{Ca}^{2+}_{\text{cyt}}$ that is mediated by CypD.

Wild-type cortical astrocytes exhibited reduced $\text{Ca}^{2+}_{\text{cyt}}$ responses, but increased glutamate release, when treated with cyclosporin A or FK506

Mitochondria devoid of CypD sequester more free Ca^{2+} than wild-type mitochondria (Fig. 2), while the agent CsA inhibits CypD to increase Ca^{2+} taken up by mitochondria [15]. To further verify the role of CypD in $\text{Ca}^{2+}_{\text{cyt}}$ response and consequential glutamate release, we inhibited CypD with CsA ($20\ \mu\text{M}$, 20 min) and observed the effects of this acute pharmacological inhibition on mechanically-induced $\text{Ca}^{2+}_{\text{cyt}}$ response and glutamate release in wild-type cortical astrocytes. In addition, we investigated whether the effect of CsA on the $\text{Ca}^{2+}_{\text{cyt}}$ responses and glutamate release of cortical astrocytes includes additional extra-mitochondrial targets. Namely, the application of CsA in astrocytes has been reported to inhibit the phosphatase calcineurin (CaN) [31,32]. In smooth muscle cells, CaN has been reported to dephosphorylate the IP_3R to decrease Ca^{2+} release from the ER [33]; this is the very store that represents the source of Ca^{2+} for glutamate release from astrocytes [5]. Additionally, CsA acts via CaN to suppress exocytosis of renin from juxtaglomerular cells [34]. In these cells $\text{Ca}^{2+}_{\text{cyt}}$ negatively modulated exocytosis of renin through calmodulin (CaM)-dependent CaN activity presumably at the level of secretory machinery.

Employing fluo-3 to measure the $\text{Ca}^{2+}_{\text{cyt}}$ response induced by mechanical stimulation, we found that wild-type astrocytes treated with CsA ($n=19$) exhibited reduced peak and cumulative responses when compared to control ($n=65$), untreated astrocytes (one-way ANOVA followed by Fisher's LSD test, $p<0.01$; Fig. 4A–C). These data are not supportive of CsA actions via CaN- IP_3R pathway since one should see an increase in the $\text{Ca}^{2+}_{\text{cyt}}$ response, but not a decrease. It should be also noted that the acute CsA treatment of wild-type astrocytes caused a greater effect on the reduction of $\text{Ca}^{2+}_{\text{cyt}}$ responses (Fig. 4B; $n=19$, normalized peak fluo-3 fluorescence = 0.69 ± 0.06) than the genetic ablation of CypD (Fig. 3B; $n=65$, normalized peak fluo-3 fluorescence = 0.92 ± 0.02) (Student t-test, $p<0.01$). Thus, although CsA is presumably acting at the MPTP opening after binding to CypD, CsA might also affect additional cyclophilins besides CypD. Nonetheless, in parallel experiments, the treatment of astrocytes with CsA ($n=12$) affected mechanically-induced glutamate release from wild-type astrocytes (Fig. 4D–F). When astrocytes were treated with CsA they displayed a significant increase in the cumulative glutamate release (one-way ANOVA, followed by Fisher's LSD test, $p<0.05$), but no significant difference was observed in peak response when compared to untreated control ($n=42$) (Fig. 4E–F). However, the peak of glutamate release due to mechanical stimulation in wild-type astrocytes treated with CsA

(Fig. 4E; $n=12$, normalized peak NADH fluorescence = 1.38 ± 0.21) was similar to that obtained from stimulated, but untreated CypD deficient astrocytes (Fig. 3E; $n=42$, normalized peak NADH fluorescence = 1.37 ± 0.13) (Student t-test, $p=0.96$). Taken together, these findings indicate the role of CypD in modulation of Ca^{2+} dependent glutamate release from astrocytes.

To test whether mechanically-induced $\text{Ca}^{2+}_{\text{cyt}}$ responses may include extra-mitochondrial signaling, more precisely modulation of ER stores, we used FK506 (1 μM , 20 min). This agent can target FKBP12 [for review of FKBP12 see [17]], a member of a PPIase/immunophilin family distinct from cyclophilins. Apart from the usual PPIase dependent activity of many other FKBP family proteins, FKBP12 can act as a scaffolding protein to anchor the phosphatase CaN, or the kinase mammalian target of rapamycin (mTOR) to IP_3R [17,35,36] or ryanodine-sensitive receptor (RyR) channels [37]. Both of these channels on the ER play a role in Ca^{2+} -dependent glutamate release from astrocytes [5]. Consequently, FKBP12 may indirectly modulate Ca^{2+} release from ER to the cytosol via two effector proteins: CaN by inhibiting the release of Ca^{2+} from ER, while mTOR potentiates it [33,38,39]. Alternatively, it has been shown in patch-clamped giant liposomes that FKBP12 protein alone may coordinate channel properties of IP_3R channels (Type 1) [40]. FKBP12 increased the fully open state of IP_3R channels, and appeared to coordinate the gating of neighboring channels. Thus, application of FK506 abolished these channel characteristics.

When examining the $\text{Ca}^{2+}_{\text{cyt}}$ responses induced by mechanical stimulation, we found that wild-type astrocytes treated with FK506 ($n=19$) exhibited reduced peak and cumulative responses when compared to control ($n=65$), untreated astrocytes (one-way ANOVA followed by Fisher's LSD test, $p<0.01$; Fig. 4A–C). Additionally, FK506 treatment of wild-type astrocytes caused a greater effect on the reduction of cumulative $\text{Ca}^{2+}_{\text{cyt}}$ responses than treatment with CsA (one-way ANOVA followed by Fisher's LSD test, $p<0.01$; Fig. 4C). These data are consistent with previous observations that FK506 inhibits the FKBP12-mediated facilitation and coordination of IP_3R channel opening [40].

In parallel experiments, treatment of astrocytes with FK506 ($n=12$) affected mechanically-induced glutamate release from wild-type astrocytes (Fig. 4D–F). Treatment of wild-type astrocytes with FK506 caused a significant increase in both the peak and cumulative mechanically-induced glutamate release (one-way ANOVA, followed by Fisher's LSD test, $p<0.01$ and $p<0.05$, respectively) (Fig. 4D–F). Taken together, these findings indicate a role for FKBP12 in modulation of Ca^{2+} -dependent glutamate release from astrocytes. Furthermore, they implicate that actions of CsA and FK506 on the $\text{Ca}^{2+}_{\text{cyt}}$ response and glutamate release of mouse cortical astrocytes may require additional extra-mitochondrial signaling.

One likely explanation for seemingly disparate findings of unparalleled changes in $\text{Ca}^{2+}_{\text{cyt}}$ and Ca^{2+} -dependent glutamate release could be that CaN in concert with its binding partner CaM exerted its/their actions directly at the level of secretory machinery, but not at the level of the ER. This Ca^{2+} /CaM-dependent phosphatase can affect vesicular proteins involved in exo/endocytosis [41] and references therein], consequently increasing the $\text{Ca}^{2+}_{\text{cyt}}$ sensitivity of secretory machinery, a process commonly referred to as down-stream of Ca^{2+} modulation of secretory machinery [42,43]. CaN by its actions at secretory machinery suppresses exocytosis, this action can be blocked by CsA or FK506 and results in enhancement of exocytosis, similar to the promotion of exocytosis seen after inhibition of CaN/CaM in juxtaglomerular cells [34]. It should be noted that in cell-free experiments, CsA-Cyp or FK506-FKBP12 complexes, but not Cyp or FKBP12, bind and inhibit CaN [44]. Of course an extension to the above enhancement concept is that besides, or

alternatively to FKBP12, there could be an involvement of large FKBP1s which are also targets of FK506 [reviewed in [17]; also see Discussion].

Inhibition of FKBP in CypD KO cortical astrocytes reduced $\text{Ca}^{2+}_{\text{cyt}}$ responsiveness, but increased glutamate release

To further elucidate whether CypD and/or $\text{IP}_3\text{R/RyR}$ channels are targets of CsA- and FK506-mediated effects on astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ dynamics and glutamate release, we evaluated mechanically-induced $\text{Ca}^{2+}_{\text{cyt}}$ responses and glutamate release of CypD KO astrocytes when treated with CsA or FK506. We found that CypD KO astrocytes treated with CsA ($n=19$; 20 μM , 20 min) exhibited some reduction in their $\text{Ca}^{2+}_{\text{cyt}}$ response (Fig. 5A–C). Analysis of the peak and cumulative $\text{Ca}^{2+}_{\text{cyt}}$ response after normalization revealed that CypD KO astrocytes treated with CsA had a reduced $\text{Ca}^{2+}_{\text{cyt}}$ peak (one-way ANOVA followed by Fisher's LSD test, $p<0.05$; Fig. 5B–C). Additionally, this reduction ($n=19$; normalized peak fluo-3 fluorescence = 0.89 ± 0.04) was significantly smaller than that recorded from wild-type astrocytes treated with CsA (Fig. 4B, $n=19$; normalized peak fluo-3 fluorescence = 0.69 ± 0.06 ; Student t-test, $p<0.02$). This suggests that CsA besides targeting CypD also has additional sites of action. In parallel experiments, CypD KO astrocytes treated with CsA ($n=12$) revealed a trend in the increase in both peak and cumulative NADH fluorescence, which did not reach significance (Fig. 5D–F). However, this trend in increase of peak and cumulative glutamate release recorded from CsA treated CypD KO astrocytes ($n=12$, Fig. 5E–F; normalized peak and cumulative NADH fluorescence = 1.32 ± 0.37 and 1.19 ± 0.22 , respectively) was similar (Student t-tests, $p=0.88$ and 0.37 , respectively) to release recorded from wild-type astrocytes treated with this agent ($n=12$, Fig. 4E–F; normalized peak and cumulative NADH fluorescence = 1.38 ± 0.21 and 1.53 ± 0.30 , respectively). Taken together these data suggest that actions of CsA in the reduction of $\text{Ca}^{2+}_{\text{cyt}}$ peak responses include non-CypD targets, perhaps other cyclophilins.

We next evaluated FK506-mediated effects on astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ dynamics and glutamate release in CypD KO astrocytes that were mechanically stimulated. Analysis of the peak and cumulative $\text{Ca}^{2+}_{\text{cyt}}$ response after normalization revealed that CypD KO astrocytes treated with FK506 had a marginally, but significantly reduced cumulative $\text{Ca}^{2+}_{\text{cyt}}$ response compared to untreated controls (one-way ANOVA followed by Fisher's LSD test, $p<0.05$; Fig. 5B–C). The peak and cumulative responses ($n=19$, Fig. 5B–C; normalized peak and cumulative fluo-3 fluorescence = 1.07 ± 0.08 and 0.84 ± 0.09 , respectively) were significantly higher than those recorded from wild-type astrocytes treated with FK506 ($n=19$, Fig. 4B–C; normalized peak and cumulative fluo-3 fluorescence = 0.69 ± 0.06 and 0.47 ± 0.06 , respectively; Student t-tests, $p<0.01$). This is an unexpected finding, since FK506 actions in wild-type astrocytes modulated $\text{IP}_3\text{R/RyR}$ via FKBP12 to significantly reduce $\text{Ca}^{2+}_{\text{cyt}}$ responses. It is possible that such activity requires interactions between ER and mitochondria containing CypD, since close contacts with IP_3Rs of the ER determines mitochondrial Ca^{2+} responses [45].

In parallel experiments, CypD KO astrocytes treated with FK506 ($n=12$) displayed significant increase in both peak and cumulative NADH fluorescence when compared to untreated control cells ($n=42$) (one-way ANOVA followed by Fisher's LSD test, $p<0.01$; Fig. 5E–F). Glutamate release recorded from FK506 treated CypD KO astrocytes ($n=12$, Fig. 5E–F; normalized peak and cumulative NADH fluorescence = 2.38 ± 0.70 and 2.44 ± 0.93 , respectively) was similar (Student t-tests, $p=0.36$ and 0.38 , respectively) to that recorded from wild-type astrocytes treated with FK506 ($n=12$, Fig. 4E–F; normalized peak and cumulative NADH fluorescence = 1.63 ± 0.38 and 1.49 ± 0.39 , respectively). Taken together, the observed acute pharmacological effects of FK506 on CypD KO, further support the notion that FKBP1s are a part of signaling transduction that mediates Ca^{2+} -glutamate release from astrocytes. Similarly, these findings are in support of the role of the

down-stream of Ca^{2+} modulation at the level of secretory machinery, perhaps via CaN. Thus, the most plausible explanation for the augmentation of glutamate release unjustified by the magnitude of $\text{Ca}^{2+}_{\text{cyt}}$ responses (Fig. 3–5) could be the phosphorylation status of the secretory machinery [[34, 41] and references therein]. It should be noted that recently has been shown that synaptosome-associated protein of 23 kDa, a part of the core secretory machinery in astrocytes [46], can be phosphorylated by the protein kinase C, which negatively regulates exocytotic glutamate release from astrocytes [47].

DISCUSSION

In this study we used mechanical stimulation that could mimic a physiological event. Mechanical stimulation activates signaling pathways, i.e., intracellular Ca^{2+} increases and consequential glutamate release, used by agonists such as ATP and bradykinin [20]; TRPC1 channels provide the conduit for Ca^{2+} entry from the extracellular space in this process [6]. Since TRPC1 was found to be a component of vertebrate mechanosensitive cation channels [48], it is feasible that mechanical transduction could be a physiological event mediated by TRPC1 channels perhaps at the astrocytic interface with blood vessels where astrocytic end feet undergo morphological changes and display changes in Ca^{2+} dynamics while these cells control microcirculation [49].

Our data show that CypD KO and wild-type astrocytes contain similar amounts of mitochondria and mitochondrial proteins, with the obvious exception of CypD KO astrocytes lacking CypD (Fig. 1). Cell-free mitochondria purified from CypD KO astrocytes have increased capacity to buffer Ca^{2+} when compared to mitochondria from wild-type astrocytes (Fig. 2), a finding that is consistent with the increased Ca^{2+} retention capacity of liver [15,50], cardiac and brain [28] mitochondria isolated from CypD KO mice. Our data further show that CypD deficiency can affect the $\text{Ca}^{2+}_{\text{cyt}}$ signals of intact cortical astrocytes as we recorded the reduced peak of $\text{Ca}^{2+}_{\text{cyt}}$ elevations evoked by mechanical stimulation (Fig. 3A), suggesting that CypD may play a significant role in astrocytic functions. In the central nervous system, CypD is more highly expressed in synaptic *versus* nonsynaptic mitochondria of neurons in various regions [51]. Furthermore, several studies have shown that mitochondria regulate $\text{Ca}^{2+}_{\text{cyt}}$ signaling and thereby affect release of neurotransmitters in central and peripheral neurons [52–54]. However, CypD is also expressed in mitochondria of astrocytes in culture and *in vivo*, albeit at lower levels [51,55]. The role of CypD and other immunophilins in Ca^{2+} -dependent gliotransmitter release was not previously studied.

In our previous study, the presumed inhibition of CypD with CsA reduced the cumulative $\text{Ca}^{2+}_{\text{cyt}}$ signal induced by mechanical stimulation in rat cortical astrocytes [7]. In the present study, genetic ablation of CypD in mouse astrocytes reduced the peak of $\text{Ca}^{2+}_{\text{cyt}}$ response due to mechanical stimulation, but had no significant effect on the cumulative $\text{Ca}^{2+}_{\text{cyt}}$ response. The difference between rat and mouse astrocytes uncovered through contrasting the results of this and our previous studies could be explained by species difference. This difference does not detract from a general conclusion that CypD can shape $\text{Ca}^{2+}_{\text{cyt}}$ responses. Indeed, when wild-type mouse astrocytes in the present study were treated with CsA, both peak and cumulative $\text{Ca}^{2+}_{\text{cyt}}$ responses were inhibited compared to control. Thus, the additional reason for differences between the acute pharmacological *versus* chronic genetic approach could be due to multiple sites of action and/or compensatory gene expression due to knock-out of CypD. To date we are unaware of transcriptome studies that chronicle compensative changes that occur in these KO mice as has been done in the case of e.g., connexin 43 [56,57]. Nonetheless, the mechanism by which CypD inhibition affects the level of $\text{Ca}^{2+}_{\text{cyt}}$ in stimulated astrocytes likely includes MPTP opening (Figs. 4A–C, and 6). Although it is thought that MPTP opening occurs at high $[\text{Ca}^{2+}]_{\text{cyt}}$ in pathological

conditions [15,58,59] there is also evidence that transient opening of the MPTP may serve a mitochondrial Ca^{2+} release channel in more physiological conditions [14]. In the present and our previous works [7] where experimental conditions involve $[\text{Ca}^{2+}]_{\text{cyt}}$ that are within the physiological, low to mid micromolar, range suggest an effect of CsA on the transient opening of the MPTP and subsequent mitochondrial Ca^{2+} release. Another important observation from the present study is that FK506 treatment reduced astrocytic $\text{Ca}^{2+}_{\text{cyt}}$ response to mechanical stimulus. Treatment of wild-type astrocytes with FK506 decreased peak and cumulative $\text{Ca}^{2+}_{\text{cyt}}$ response (Fig. 4A–C). A likely mechanism is that FK506 directly inhibits FKBP12 facilitation and coordination of IP₃R channel openings (Fig. 6), as has been shown in giant liposomes [36].

Ca^{2+} -dependent glutamate release in astrocytes has a strict relationship to $\text{Ca}^{2+}_{\text{cyt}}$ response [30] with the increase in $\text{Ca}^{2+}_{\text{cyt}}$ being sufficient and necessary for this release to occur [24]. Hence, in our previous study, CsA which caused the decrease in $\text{Ca}^{2+}_{\text{cyt}}$ response in mechanically stimulated rat astrocytes resulted in the reduced glutamate release [7]. However, and perhaps further pointing some species differences, the present study provides evidence that CypD deficient mouse astrocytes exhibit augmented release of glutamate compared to wild-type while exhibiting blunted $\text{Ca}^{2+}_{\text{cyt}}$ responses to stimuli (Fig. 3). This finding was echoed by the effects of CsA and FK506 on wild type (Fig. 4) and CypD KO (Fig. 5) astrocytes. Interestingly, CsA has been found to act via CaN to suppress exocytosis of renin from juxtaglomerular cells [34], so that $\text{Ca}^{2+}_{\text{cyt}}$ negatively modulated exocytosis of renin through calmodulin (CaM)-dependent CaN activity presumably at the level of secretory machinery. This or a similar mechanism appears to be the most plausible explanation for our observation of down-stream of Ca^{2+} modulation of Ca^{2+} -dependent glutamate release from astrocytes that was mediated by immunophilins CypD and FKBP12 (Fig. 6.).

In primary hippocampal neurons, inhibition of CaN with FK506 and CsA had various effects on the endocytic pathway as visualized using recycling dyes [41]. This effect likely occurred due to the (de)phosphorylation status of endo/exocytotic proteins. Inhibition of CaN suppressed recycling of vesicles within their reserve, but not the ready-releasable pool. Additionally, higher (10 Hz), rather than lower (3–5 Hz), frequency stimulation-induced exocytosis was reduced by the inhibition of CaN, as the total pool of vesicles was reduced. Based on our data using CsA and FK506 to affect astrocytic intracellular Ca^{2+} dynamics and exocytotic glutamate release, it is tempting to hypothesize that the down-stream of Ca^{2+} modulation of glutamate release from astrocytes would have consequences on synaptic transmission and plasticity at the tripartite synapse.

Although the effects of FK506 observed herein are intuitively attributed to FKBP12 (encoded by *FKBP1A* gene) tampering with IP₃R/RyR channels of the ER and CaN/CaM at the level of secretory machinery, it is feasible that other large FKBP's were also affected. The large FKBP52 (encoded by *FKBP4* gene) is found to co-localize with the cellular cytoskeleton [60], in cell-free conditions can bind CaM [61] and the microtubule-associated protein Tau [62], as well as it can associate in cell lysates with dynein [63], a major protein involved in retrograde trafficking along microtubules. Another large FK506-binding protein, FKBP51 (encoded by *FKBP5* gene) can precipitate purified brain CaN in a cell-free environment [64].

Interestingly, when comparing protein and gene expression of FKBP51 and FKBP52 in autopsy tissue obtained from frontal cortex gray matter, there was a correlation between human immunodeficiency virus (HIV)-infected patients and up regulated levels of both large immunophilins [65]. HIV-infected patients have a higher risk of developing major depressive disorder (MDD) [66]. Indeed, in HIV positive patients with MDD there was an

increased *FKBP4* expression. The level of *FKBP5* expression and FKBP51 correlated with MDD alone, with additionally increased frequency for the *FKBP5* single nucleotide polymorphism (SNP). The polymorphism of *FKBP5* has also been associated with the increase rate of depressive episodes and rapid response to antidepressant treatment [67]. Furthermore, *FKBP5* in humans has been implicated in etiology of posttraumatic stress disorder (PTSD) and depression. For instance, SNPs in *FKBP5* has been found as predictor for severity of adult PTSD if individuals were abused during their childhood [68]. In individuals with current PTSD, *FKBP5* showed reduced expression [69]. Whether large FKBP5s are underlying some of the effects seen in our study and whether exocytotic glutamate release from astrocytes could be involved in the above pathologies and serve as a cellular substrate for their treatments may turn out to be a valuable venture to explore.

Our findings of augmented glutamate release from astrocytes could be important in etiology/progression of Alzheimer's disease. Changes in astrocytes, including simultaneous astroglial atrophy and astrogliosis, represent an integral part of this neurodegenerative disease [70,71]. A recent study of mice used as a model for Alzheimer's disease (mAPP mice) showed improved long-term potentiation (LTP) and spatial learning and memory when the CypD gene was deleted [72]. While this study does not further specify if the improvement in LTP in mAPP mice lacking CypD is due to changes in presynaptic or postsynaptic elements, our data showing that CypD deficient cortical astrocytes have augmented glutamate release suggest that astrocytes, as a third component of synapses, may be involved in this improvement by modulation of glutamatergic synaptic transmission and LTP [73–75]. Another study suggests that astrocytes from mAPP mice exhibited elevated Ca^{2+} levels and aberrant Ca^{2+} wave activity [76], although it has not been established whether astrocytes from these animals exhibited changes in astrocytic glutamate release. Thus, investigations of the role of astrocytic glutamate release, with their mitochondria as a source/sink for $\text{Ca}^{2+}_{\text{cyt}}$ necessary for exocytotic release together with immunophilin-dependent effects on this release, are warranted in Alzheimer's disease models, since astrocytes may prove to be a site for medical treatment. In conjunction with this devastating disease, but also more generally related to physiology of the brain, it would be important to address whether immunophilin- and FK506/CsA-dependent effects on astrocytic glutamate release exhibit regional (e.g., hippocampus *versus* cortex) and age-related differences.

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Figure 1.

Cultured astrocytes from wild-type (WT) and cyclophilin D knock-out (CypD KO) mice contain similar amounts of mitochondria. A) Astrocytic mitochondria were labeled with 4-Di-2-ASP (top panels), while their cytoplasm was revealed using the vital stain calcein (bottom panels). Scale bar, 20 μ m B) The area of the cell occupied by mitochondria was unchanged by the deletion of CypD, since there was no significant difference between obtained ratios of 4-Di-2-ASP/calcein positive pixels from wild-type and CypD KO astrocytes. C) Immunoblot analysis of cellular lysates from wild-type and CypD KO astrocytes did not reveal major differences in the expression levels of key mitochondrial proteins localized in the matrix (ATP synthase), inner (ATP synthase and complex II subunits) and outer (porin 31HL) membranes of the organelle. Note the absence of the matrix protein CypD in lysates from CypD KO astrocytes. Immunoreactivity of tubulin was used as a loading control.

Figure 2.

The absence of CypD increases Ca^{2+} uptake capacity of astrocytic mitochondria. A) Traces represent kinetics of Ca^{2+} uptake by mitochondria isolated from astrocytes of WT and CypD KO mice. Mitochondrial Ca^{2+} capacities were monitored in a suspension of mitochondria (0.5 mg/ml) by examining the changes in extramitochondrial Calcium Green-5N fluorescence upon Ca^{2+} additions (spikes; 10 nmol per 2 μl aliquot). Increases in fluorescence reflect increases in extra mitochondrial Ca^{2+} , whereas decreases in fluorescence reflect a mitochondrial Ca^{2+} uptake. After a limited number of Ca^{2+} additions, mitochondria from wild-type astrocytes fail to uptake Ca^{2+} (WT, top trace). In contrast, mitochondria isolated from CypD KO astrocytes were able to uptake Ca^{2+} more efficiently and to buffer a substantially higher amount of Ca^{2+} (CypD KO; lower trace) when compared to WT. B) Immunoblot analysis show similar levels of ATP synthase and porin 31HL in the mitochondrial suspensions from WT and CypD KO astrocytes after experiments in (A), confirming equal loads of organelles in the fluorescence assay. Note the absence of CypD in mitochondrial suspensions obtained from CypD KO astrocytes.

Figure 3.

Cultured cortical astrocytes from CypD KO mice exhibit reduced cytosolic Ca^{2+} ($\text{Ca}^{2+}_{\text{cyt}}$) peak response, but exhibit augmented glutamate release in response to mechanical stimulation when compared to cortical astrocytes from WT mice. A) Average kinetics of the fluo-3 fluorescence indicating the change in $\text{Ca}^{2+}_{\text{cyt}}$. B) Normalized fluo-3 peak fluorescence values of mechanically-stimulated astrocytes. CypD KO astrocytes had significantly lower peak fluorescence than WT control C) Normalized fluo-3 cumulative fluorescence values obtained from mechanically-stimulated astrocytes. D) Average kinetics of the NADH fluorescence reporting on glutamate released by astrocytes. E–F) Normalized NADH peak (E) and cumulative (F) fluorescence values of mechanically-stimulated astrocytes. CypD KO astrocytes display significantly higher peak and cumulative NADH fluorescence than WT control astrocytes. Points and bars represent means $\pm$ SEMs of measurements from individual solitary astrocytes (numbers for A–C and D–F listed in parentheses of A and D, respectively) expressed as dF/Fo (percentage) or normalized (ratio to control), while arrows (in A and D) represent the time when mechanical stimulation was applied to the cells. Asterisks denote significant change in comparison to control (WT) group (Mann-Whitney U-test, * $p < 0.02$, ** $p < 0.01$).

Figure 4.

Cyclosporin A and FK506 reduce $\text{Ca}^{2+}_{\text{cyt}}$ responses of wild-type cortical astrocytes, but increase glutamate release from these cells evoked by mechanical stimulation. A) Average kinetics of the fluo-3 fluorescence in wild-type cortical astrocytes untreated with pharmacological agent (WT) or treated with cyclosporin A (CsA; 20 μM , 20 min), or FK506 (1 μM , 20 min). B–C) When normalized to control, untreated WT astrocytes, the peak and cumulative fluo-3 fluorescence of astrocytes treated with CsA, or FK506 are significantly lower than the control. Also, FK506 shows a significant decrease in cumulative fluo-3 fluorescence when compared to CsA treated astrocytes. D) Average kinetics of the NADH fluorescence reporting on glutamate released by WT astrocytes untreated with agent or treated with CsA or FK506. E–F) Normalized NADH peak (E) and cumulative (F) fluorescence values of mechanically-stimulated astrocytes. Wild-type astrocytes when treated with CsA or FK506 display an increased glutamate release. Points, bars and arrows as described in Fig. 3. Asterisks denote significant change in comparison to control group (one-way ANOVA, followed by Fisher's LSD test, * $p < 0.05$, ** $p < 0.01$). Note also the comparison between CsA and FK506 groups in C.

Figure 5.

Cyclosporin A and FK506 reduce Ca^{2+}_{cyt} responses, but only FK506 augmented glutamate release from CypD KO cortical astrocytes evoked by mechanical stimulation. A) Average kinetics of the fluo-3 fluorescence reporting on Ca^{2+}_{cyt} in CypD KO cortical astrocytes untreated with pharmacological agent or treated with CsA (20 μ M, 20 min), or FK506 (1 μ M, 20 min). B–C) When normalized to control (solitary untreated CypD KO astrocytes), the peak and cumulative fluo-3 fluorescence of astrocytes treated with CsA or FK506 show significant decreases in Ca^{2+}_{cyt} . D) Average kinetics of the NADH fluorescence reporting on glutamate released by CypD KO astrocytes untreated with an agent or treated with CsA or FK506. E–F) Normalized NADH peak (E) and cumulative (F) fluorescence values of mechanically-stimulated solitary astrocytes. Treatment of CypD KO with FK506 causes the increase in glutamate release. Points, bars and arrows as described in Fig. 3. Asterisks denote significant change in comparison to control group (one-way ANOVA, followed by Fisher'sLSD test, * $p < 0.05$, ** $p < 0.01$).

Figure 6.

Immunophilins modulate Ca^{2+} -dependent vesicular glutamate release from astrocytes. $\text{Ca}^{2+}_{\text{cyt}}$ handling can be affected by the endoplasmic reticulum (ER) and mitochondria. Store specific Ca^{2+} -ATPase (re)fills the ER with Ca^{2+} . Accumulation of $\text{Ca}^{2+}_{\text{cyt}}$ could be caused by the entry of Ca^{2+} from the internal stores of the ER via IP_3 and ryanodine receptors, which bind the scaffolding protein FKBP12. FK506 can bind to FKBP12 which leads to the reduction of Ca^{2+} efflux from the ER and $\text{Ca}^{2+}_{\text{cyt}}$ decrease. Mitochondrial Ca^{2+} uptake is mediated by the uniporter. Free Ca^{2+} exits the mitochondrial matrix through the $\text{Na}^+/\text{Ca}^{2+}$ exchanger or through the formation of the mitochondrial permeability transition pore (MPTP). $\text{Ca}^{2+}_{\text{cyt}}$ is decreased during increased Ca^{2+} buffering by mitochondria caused by cyclosporin A (CsA) that can bind to CypD to prevent formation of the MPTP. An increase in $\text{Ca}^{2+}_{\text{cyt}}$ is sufficient and necessary to cause vesicular fusions and release of glutamate, a process that requires exocytotic secretory machinery (not shown). Secretory machinery could be negatively modulated by calcineurin (CaN) and Ca^{2+} /calmodulin (dotted line). Here $\text{Ca}^{2+}_{\text{cyt}}$ decrease caused by FK506 and CsA actions at their ER and mitochondrial targets, respectively, would then result in the reduction on the negative modulation at the secretory machinery. Similarly, CaN at release sites could be blocked by FK506 or CsA (dashed lines). Both events would result in the augmented glutamate release from astrocytes. Drawing is not to scale.