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A Single Amino Acid Determines the Selectivity and Efficacy of Selective Negative Allosteric Modulators of Ca_v1.3 L-Type Calcium Channels

Garry Cooper^{1,2,3}, Soosung Kang^{1,3,4}, Tamara Perez-Rosello², Jaime N. Guzman², Daniel Galtieri², Zhong Xie², Jyothisri Kondapalli², Jack Mordell², Richard B. Silverman^{*,1,3,5}, D. James Surmeier^{*,2}

¹Department of Chemistry, Northwestern University, Evanston, IL 60208-3113 USA

²Department of Physiology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611 USA

³Chemistry of Life Processes Institute, Center for Molecular Innovation and Drug Discovery, Center for Developmental Therapeutics, Northwestern University, Evanston, IL 60208 USA

⁴College of Pharmacy, Ewha Womans University, Seoul, Korea

⁵Department of Pharmacology, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611 USA

Abstract

Ca²⁺ channels with a Ca_v1.3 pore-forming α_1 subunit have been implicated in both neurodegenerative and neuropsychiatric disorders, motivating the development of selective and potent inhibitors of Ca_v1.3 vs Ca_v1.2 channels, the calcium channels implicated in hypertensive disorders. We have previously identified pyrimidine-2,4,6-triones (PYTs) that preferentially inhibit Ca_v1.3 channels, but the structural determinants of their interaction with the channel have not been identified, impeding their development into drugs. Using a combination of biochemical, computational, and molecular biological approaches, it was found that PYTs bind to the dihydropyridine (DHP) binding pocket of the Ca_v1.3 subunit, establishing them as negative allosteric modulators of channel gating. Site-directed mutagenesis, based on homology models of Ca_v1.3 and Ca_v1.2 channels, revealed that a single amino acid residue within the DHP binding pocket (M1078) is responsible for the selectivity of PYTs for Ca_v1.3 over Ca_v1.2. In addition to providing direction for chemical optimization, these results suggest that, like dihydropyridines, PYTs have pharmacological features that could make them of broad clinical utility.

Graphical Abstract

*Corresponding Authors RBS: Tel: 847-491-5653. Agman@chem.northwestern.edu, DJS: Tel: 312-503-4904. j-surmeier@northwestern.edu.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:

Synthesis of [³H]-D11, current-voltage relationships of Ca_v1.3/Ca_v1.2 mutation constructs in domains III and IV, Ca_v1.3 and Ca_v1.2 homology models with cp-PYT docked in to show repulsive effects

Keywords

Ca_V1.3; L-type calcium channels; pyrimidine-2,4,6-triones; Parkinson's disease; dihydropyridines; homology model; site-specific mutagenesis

Plasma membrane, voltage-dependent Ca²⁺ channels regulate a wide range of cellular functions throughout the body. These channels are heteromeric proteins, consisting of a pore-forming α_1 subunit and auxiliary subunits. These subunits dictate channel gating, trafficking, localization, and coupling with intracellular signaling cascades.¹ Because of its centrality to channel function and pharmacology, Ca²⁺ channels are divided into classes based on their pore-forming α_1 subunit. One these classes – L-type – has a pore-forming Ca_V1 α_1 subunit; four genes have been cloned (*CACNA1A-D*).² In the brain, channels with a Ca_V1.2 (*CACNA1C*) subunit predominate, consisting of roughly 90% of all L-type Ca²⁺ channels, with Ca_V1.3 (*CACNA1D*) channels making up almost all of the remainder.³

Although Ca_V1 channels are commonly considered ‘high-voltage activated’ because their open probability increases only at membrane potentials above spike threshold in neurons, Ca_V1.3 channels have an activation voltage-dependence that is very similar to that of ‘low threshold’ Ca²⁺ channels that open at membrane potentials below or near spike threshold.⁴ As a consequence, the functional roles played by Ca_V1.3 channels appear to be distinct from those of high-threshold Ca_V1.2 channels. For example, in the brain Ca_V1.3 channels have been implicated in the genesis of autonomous pacemaking and integration of synaptic events underlying plasticity.^{5, 6, 7, 8} They also have been linked to a wide variety of psychomotor disorders, including Parkinson's disease (PD), autism spectrum disorder (ASD), bipolar disorder, and drug abuse.^{6, 9, 10, 11} Ca_V1.2 channels, on the other hand, also are abundant in peripheral organs, including the heart, and are the target of a variety of antihypertensive agents.¹² Poor selectivity of compounds for Ca_V1.3 over Ca_V1.2 channels leads to undesirable cardiovascular side effects.³

Despite the growing body of evidence linking Ca_V1.3 Ca²⁺ channels to human disease, a potent and selective inhibitor of Ca_V1.3 channels that might be suitable for clinical use has not been developed. At present, the only class-specific inhibitors of Ca_V1.3 channels approved for human use are 1,4-dihydropyridines (DHPs); however, DHPs are potent inhibitors of both Ca_V1.2 and Ca_V1.3 channels, with most members of this drug class

exhibiting a 5–10-fold higher affinity for Ca_v1.2 channels.³ A key feature of DHPs is that they are negative allosteric modulators (NAMs) of Cav1 channel gating, rather than being pore-blockers.¹ This confers voltage-dependence to their action, as their affinity is several orders of magnitude lower at membrane potentials at which most neurons rest, increasing into the nanomolar range with depolarization and activity. This ‘use-dependence’ is undoubtedly critical to their clinical efficacy and minimal side-effect profile.

Given the clinical and scientific rationale for a Ca_v1.3-selective inhibitor, some years ago our group undertook a compound screening effort. Starting from a conventional Ca²⁺ fluorescence-based counter-screen against Ca_v1.2 and Ca_v1.3 channels, a pyrimidine-2,4,6-trione (PYT) was found to show a slight preference for Ca_v1.3 channels over Ca_v1.2 channels. Multiple cycles of lead optimization of the initial hit compound eventually led to 1-(3-chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-trione (cp-PYT), which was found to be highly selective for inhibition of Ca_v1.3 channels over Ca_v1.2 channels.¹³ However, it was unclear where on the channel cp-PYT bound. The fact that the auxiliary subunits of the Ca_v1.2 and Ca_v1.3 channels used in the assay were the same clearly implicated the pore-forming α_1 subunit. But it was unclear where on the α_1 subunit that binding took place.

Subsequent to our initial report, two papers appeared that raised questions about the potential value of cp-PYT (and its derivatives) as Ca_v1.3 channel inhibitors. In the first paper, Ortnier *et al.*¹⁴ reported that micromolar concentrations of cp-PYT slowed the deactivation of tail currents, essentially enhancing – not inhibiting – currents through Ca_v1.3 channels. In the second paper, Huang *et al.*¹⁵ reported that cp-PYT did inhibit Ca_v1.3 channels, but that the magnitude and selectivity of the inhibition was dependent on the auxiliary β subunit, not the α_1 subunit.

The present studies were designed to determine the site of binding of cp-PYT to Ca_v1.3 channels, which should allow future rational design of more potent and selective NAMs of Ca_v1.3, thereby minimizing the undesirable cardiovascular effects of Ca_v1.2 binding. In addition, these studies clarified the above-noted literature discrepancies as well. To this end, a combination of approaches, including site-directed mutagenesis, equilibrium radiolabeled binding studies, and computer modeling, were used to define the cp-PYT binding site on heterologous Ca_v1.3 channels. In addition, the effects of cp-PYT on natively expressed Ca_v1.3 channels in *substantia nigra* dopaminergic neurons were determined. These studies revealed that cp-PYT binds to the same region of the α_1 subunit as DHPs. Homology modeling and site-directed mutagenesis studies demonstrated that the selectivity of cp-PYT for Ca_v1.3 channels at this site was dependent on a single amino acid residue. As with DHPs, cp-PYT binding to this site and inhibition of channel opening was voltage-dependent, making cp-PYT a NAM, like DHPs. From these studies, it was realized that the previously reported study that failed to see cp-PYT inhibition¹⁴ used a variant of the Ca_v1.3 subunit lacking this key single amino acid residue, thereby abolishing the activity of cp-PYT. In addition to this high affinity binding site, a lower affinity site was found. Binding of cp-PYT to this lower affinity site led to slowing of tail current kinetics and could confer a dependence upon the α_1 subunit used to form the channel, as noted by earlier investigators.¹⁵ Collectively, these studies not only provide a roadmap for the development of more potent

and selective Ca_v1.3 channel NAMs that could be of clinical utility, they also provide explanations for the discrepancies with previous studies.

RESULTS

Site-directed mutagenesis of the DHP binding site disrupted PYT inhibition

Ca_v1 channels are known to be inhibited by three drug classes: 1,4-dihydropyridines (DHPs), phenylalkylamines, and benzothiazepine. These three classes have overlapping and allosterically-coupled binding sites^{16, 17} The best characterized of these sites is the DHP binding site. As a first step toward determining if PYTs interact with this site, a known key residue in this binding pocket was mutated, and the effects of this mutation on the ability of cp-PYT to inhibit channel currents were assessed. In the Ca_v1.2 subunit, T1066 is critical to DHP binding and channel inhibition.^{18, 19, 20} Mutating this amino acid to tyrosine disrupts DHP binding.¹⁹ The homologous amino acid in the Ca_v1.3 subunit is T1081 (Fig. 1a,b). Therefore, to determine the effect on DHP and PYT the corresponding threonine in Ca_v1.3 was mutated. This site in the Ca_v1.2 subunit is well established to determine DHP sensitivity.³ Mutating this threonine residue to a tyrosine (T1081Y) had no effect on Ca_v1.3 channel gating or current when expressed in HEK293 cells with auxiliary β 3 and α 2 δ subunits (**data not shown**). However, this substitution significantly reduced the inhibition of evoked currents by a saturating concentration of the DHP isradipine (5 μ M) (Fig. 1c,d).²¹ This mutation also significantly diminished the inhibition of evoked currents by cp-PYT at a nominally saturating concentration (100 μ M) (Fig. 1c,d).

The ability of the T1081Y mutation to diminish cp-PYT inhibition of Ca_v1.3 channel currents suggests that PYTs bind at the DHP site. To support this hypothesis, a tritiated version of one of our most potent PYT compounds (³H₂-**D11**)¹³ was synthesized (Supporting Information Fig. S1) and equilibrium binding experiments were conducted with membrane fractions from HEK293 cells expressing functional Ca_v1.3 channels (Fig. 1e).¹³ The binding affinity of (³H₂-**D11**) in membrane fractions was several-fold higher than that seen in intact cells (Fig. 1f,g), potentially because binding was voltage-dependent, as was the case for DHPs (membrane fractions were nominally at 0 mV in contrast to intact cells). Importantly, isradipine displaced (³H₂-**D11**) binding (Fig. 1h), suggesting that PYTs and DHPs bind to physically close sites. Isradipine failed to displace a small fraction of the (³H₂-**D11**) binding (~20%), suggesting that there was another minor PYT binding site. Our experimental system was calibrated prior to binding experiments using isradipine; a linear dependence of channel protein concentration with the specific binding of tritiated isradipine was observed (Fig. 1i).

Structural determinants of PYT binding to the Ca_v1.3 subunit

To explore the structural determinants of PYT binding to the DHP site,²² the recently published structural data for the Ca_v1.1 channel²³ was used to construct homology models of Ca_v1.2 and Ca_v1.3 channels.²⁴ The mapping of protein sequences in 3-D space was achieved using the Homology Modeling module, and the energy minimization of the produced structures was performed using the Prime module (OPLS3e force field), in Schrödinger software. Compound docking studies were then performed using the Induced

Fit Docking module in the Schrödinger software. In these studies, both isradipine and cp-PYT were found to fit into the DHP binding pocket ringed by M1078, T1081, T1082, Q1085, F1154, S1156, Y1194, V1198, M1212, I1205, F1209, I1489, Y1492, and M1493 (Fig. 2a, b). Of those surrounding amino acids, Q1085, F1154, V1198, M1212, and M1493 are known to affect DHP binding.^{18–20} As shown in Fig. 2a, the benzooxadiazole oxygen of the isradipine faces S1156, and one ester on the pyridine ring of isradipine interacts with the polar residue T1082. In the case of cp-PYT, two of the three carbonyl groups interact with polar residues T1081, Q1085, and S1156 (Fig. 2b). However, the prominent interaction of cp-PYT is the hydrophobic interaction between the side chains of cp-PYT (cyclopentyl and 3-chlorophenethyl groups) and the hydrophobic residues of Cav1.3 (M1078, F1154, V1198, I1205, F1206, F1209, M1493, and F1497). In contrast to isradipine, cp-PYT binding to Cav1.3 is influenced by the interaction with M1078 and V1198 in the DHP binding pocket of the Cav1.3 model (Fig. 2b). M1078 and V1198 of Cav1.3 are the only residues that differ from Cav1.2 in that binding site.

To test whether these two amino acids account for the selective inhibition of Cav1.3 channels, two sets of site-directed mutagenesis experiments were performed. First, the Cav1.3 subunit was mutated to mimic the Cav1.2 subunit by 1) replacing Met at 1078 in domain III-S5 of the Cav1.3 subunit with a Val, which is found at corresponding site 1063 in the Cav1.2 subunit and 2) replacing Val at 1198 in domain IV-S6 of the Cav1.3 subunit with Ile found at corresponding site 1183 of the Cav1.2 subunit (Fig. 2c). Second, analogous mutations were made in the Cav1.2 subunit to make them resemble the Cav1.3 subunit, namely, V1063 in the Cav1.2 subunit was replaced with a Met, and I1183 in the Cav1.2 subunit was replaced with a Val. The ability of isradipine and PYTs to inhibit these mutated channels was assessed by expressing the channels in HEK293 cells and performing patch clamp experiments with Ba²⁺ as the charge carrier. All of the channel mutants were fully functional and yielded robust patch clamp currents (Supporting Information Fig. S2). The ability of cp-PYT to inhibit Cav1.3 channel currents was dramatically reduced by the M1078V mutation (Fig. 2d,e), i.e., giving it Cav1.2 binding site character. In contrast, it was not significantly altered by the V1198I mutation (Fig. 2e). None of these mutations affected inhibition by isradipine at saturating concentrations (Fig. 2e). Conversely, the V1063M mutation in the Cav1.2 channel significantly enhanced cp-PYT inhibition, i.e., giving Cav1.2 more Cav1.3 binding site character, but the I1183V mutation did not (Fig. 2d,f). Again, none of these mutations significantly altered the ability of saturating concentrations of isradipine to inhibit channel currents (Fig. 2f). These data strongly suggest that M1078 in the Cav1.3 subunit is critical to the selective inhibition of channel currents by cp-PYTs.

Why is M1078 in the Cav1.3 subunit so important? Our previous work¹³ suggested that the relative size of the accessible side chains of M1078 (and V1063 in the Cav1.2 subunit) might be important for PYT binding. To determine if this is the case for cp-PYT, two sets of experiments were performed. First, M1078 in the Cav1.3 subunit was replaced with amino acids having progressively less sterically hindering side chains (Fig. 3a,b). Second, V1063 in the Cav1.2 subunit was exchanged with amino acids with progressively larger side chains (Fig. 3c). These experiments revealed a correlation between the size of the amino acid side chain at either position 1078 in Cav1.3 or 1063 in Cav1.2 LTCCs and cp-PYT inhibition

(Fig. 3d). In contrast, these mutations had no effect on the inhibition of currents by isradipine (Fig. 3e).

PYT inhibition of Ca_v1.3 channels is voltage-dependent

A key feature of DHP inhibition of Ca_v1 channels is its voltage-dependence. At depolarized membrane potentials, DHPs are several orders of magnitude more potent than at hyperpolarized membrane potentials.^{5, 25} Although the determinants of this voltage-dependence have not been completely resolved, conformational changes in the shape of the DHP binding pocket are likely to be responsible for the shift in affinity. Given that PYTs bind in the DHP pocket, their inhibition of Ca_v1.3 channels could be voltage-dependent as well. To test this hypothesis, Ca_v1.3 channel currents in HEK293 cells were studied using voltage-clamp techniques. Cells were held at either −80 mV or −50 mV prior to stepping to 0 mV to open channels. At relatively hyperpolarized holding potential (−80 mV), isradipine (5 μM) modestly inhibited peak currents, but this inhibition was dramatically increased by holding at −50 mV, as expected from previous studies (Fig. 4a). As predicted by the hypothesis that cp-PYTs interact with the DHP pocket, the ability of cp-PYT (10 μM) to inhibit Ca_v1.3 channel currents also was voltage-dependent and increased significantly at depolarized holding potentials (Fig. 4b–d).

PYT binding to a low affinity site slows channel deactivation

As shown above, isradipine failed to displace a portion of the PYT (i.e., D11) binding (Fig. 1h), suggesting that there was a second low affinity PYT binding site on the Ca_v1.3 channel. To test this hypothesis, the effects of low (10 μM) and high (50 μM) concentrations of cp-PYT on channel functioning were studied. At low concentration, cp-PYT decreased the Ca_v1.3 channel currents evoked by a voltage step to 0 mV in HEK 293 cells, but did not alter tail current kinetics or channel deactivation rates when the voltage was stepped back to −80 mV (Fig. 5a–c). However, at high cp-PYT concentration, not only was the current evoked by a depolarizing step diminished, but tail current kinetics slowed (Fig. 5d–f). These results support the proposition that slowing of deactivation is dose-dependent. To determine if this slowing of deactivation kinetics relies on binding to the DHP site, these experiments were repeated in HEK293 cells expressing the M1078V mutation, which disrupted cp-PYT binding to the DHP pocket. In these mutant channels, the effect of cp-PYT (100 μM) on the peak current was lost (Fig. 2d), but the slowing of tail currents persisted (Fig. 5g,h), suggesting that the second binding site was elsewhere on the channel. Together, these data show that deactivation kinetics are slowed only at high concentrations of cp-PYT, and this slowing is independent of the DHP binding site, clearly suggesting that there was a second, low affinity binding site that was responsible for the cp-PYT effects on channel deactivation.

PYTs diminish Ca²⁺ currents and mitochondrial oxidant stress in dopaminergic neurons

As outlined above, native Ca_v1.3 channels are heterogeneous. They can differ not only in auxiliary subunits, but also in the alternative splicing of the Ca_v1.3 subunit itself.^{26, 27, 28} Moreover, channel proteins are post-translationally modified, most notably by phosphorylation.²⁹ To assess the ability of cp-PYT to inhibit native Ca_v1.3 channels, dopaminergic neurons in the *substantia nigra* (SN) of mice were studied. These neurons robustly express Ca_v1.3 Ca²⁺ channels that give rise to large oscillations in cytosolic Ca²⁺

concentration and mitochondrial oxidant stress.^{5, 30, 31} As a first step in this analysis, SN dopaminergic neurons were acutely isolated from brain slices obtained from young adult mice to allow channels to be voltage-clamped (Fig. 6a, left). Cell-attached recordings from these cells revealed that they exhibited the autonomous activity typical of this cell type (Fig. 6a, right). In whole cell recordings with Ba^{2+} as the charge carrier, voltage ramps from -70 mV to $+30$ mV yielded currents with typical voltage dependence.³² Application of a low concentration ($5 \mu\text{M}$) of cp-PYT produced a modest inhibition of currents that was increased by elevating the concentration ($50 \mu\text{M}$) (Fig. 6b). Because several channel types contribute to these whole cell currents,^{32, 33} the experiment was redone in SN dopaminergic neurons from a knockout mouse in which the gene coding for the pore-forming subunit of the $\text{Ca}_v1.3$ channel (*CACNAD1*) had been deleted.³⁴ In these cells, cp-PYT ($50 \mu\text{M}$) had no effect (Fig. 6c,d), indicating that cp-PYT was selectively inhibiting $\text{Ca}_v1.3$ channels.

As the majority of plasma membrane $\text{Ca}_v1.3$ channels in SN dopaminergic neurons are positioned in dendrites, two photon laser scanning microscopy (2PLSM) and whole cell patch clamping were used in ex vivo brain slices from young adult mice to assess the impact of cp-PYT on dendritic Ca^{2+} channels. After dialysis of the Ca^{2+} dye Fluo-4 ($100 \mu\text{M}$) with the patch electrode, distal dendrites were imaged while SN dopaminergic neurons were pacemaking (Fig. 6e). Application of cp-PYT ($50 \mu\text{M}$) to the slice had no effect on pacemaking, but significantly reduced the dendritic Ca^{2+} oscillations, consistent with an inhibition of $\text{Ca}_v1.3$ channel opening (Fig. 6e,g).³⁰ Overlaying the dendritic fluorescence signals from before and after cp-PYT application revealed that the greatest inhibition occurred during the slow, depolarizing phase prior to spike initiation (Fig. 6f), an observation consistent with the ability of $\text{Ca}_v1.3$ channels to activate at hyperpolarized membrane potentials.^{4, 21, 30} Ca^{2+} entry through Ca_v1 channels in SN dopaminergic neurons triggers ryanodine receptor-dependent release of Ca^{2+} from intracellular stores and stimulation of mitochondrial oxidative phosphorylation (OXPHOS).³⁵ One by-product of increased OXPHOS is the production of reactive oxygen species (ROS) that can be monitored using a genetically- encoded, mitochondrially-targeted, redox-sensitive variant of green fluorescent protein (mito-roGFP) (Fig. 6h).^{30, 31, 35, 36} In ex vivo brain slices from a mouse expressing mito-roGFP under control of a tyrosine hydroxylase promoter fragment (to limit expression to dopaminergic neurons), application of isradipine ($1 \mu\text{M}$) diminished mitochondrial oxidant stress (Fig. 6i, j), as reported previously.^{30, 31} Bath application of cp-PYT ($50 \mu\text{M}$) also significantly diminished mitochondrial oxidant stress in SN dopaminergic neurons (Fig. 6i, j), consistent with the proposition that it inhibited native $\text{Ca}_v1.3$ channels linked to mitochondrial OXPHOS.³⁰

DISCUSSION

The rationale for a $\text{Ca}_v1.3$ channel selective inhibitor

Although they constitute a relatively small percentage ($\sim 10\%$) of brain Ca_v1 Ca^{2+} channels, $\text{Ca}_v1.3$ channels have been implicated in a variety of psychomotor disorders,^{6, 9, 10, 11} the best characterized of these is Parkinson's disease (PD). The cardinal motor symptoms of PD are caused by the loss of SN dopaminergic neurons.¹¹ Studies in mouse and human models have implicated $\text{Ca}_v1.3$ Ca^{2+} channels in mitochondrial dysfunction thought to contribute to

the degeneration of these neurons.¹¹ Buttredding this conclusion, epidemiological studies have shown that the treatment of hypertension with DHP, but not other hypertension medication, significantly lowers the risk of developing PD.³⁷ This combination of experimental and clinical findings led to the Phase 3, disease-modification trial in early stage PD patients with the DHP isradipine (STEADY-PD).^{37, 38} Isradipine was chosen for this trial because it had the highest relative affinity for Ca_v1.3 channels among clinically approved DHPs.³ However, because of its cardiovascular effects, isradipine dosing in PD patients was limited and not optimal for inhibition of Ca_v1.3 channels.³⁷ As the distribution of Ca_v1.3 channels is much more narrow than that of Ca_v1.2 channels,³ a Ca_v1.3-selective inhibitor is expected to be much more effective for slowing progression of PD than a DHP.

There are other clinical reasons to pursue a selective inhibitor of Ca_v1.3 channels. Ca_v1.3 channels in ventral tegmental area dopaminergic neurons have been implicated in the synaptic events underlying drug addiction, and administration of either isradipine or cp-PYT has been shown to disrupt the reinstatement of alcohol- and cocaine-seeking in rodents.⁹ In addition, gain of function mutations in Ca_v1.3 channels have been implicated in autism spectrum disorder and epilepsy,^{10, 39} further broadening the therapeutic value of a Ca_v1.3 selective inhibitor.

From a translational perspective, the fact that cp-PYT is a NAM, rather than a channel pore blocker, is significant. Undoubtedly, the modest side-effect profile of DHPs stems from their being NAMs, because indiscriminate blockage of Ca_v1.2/Ca_v1.3 channels would be lethal. Their preferential interaction with channels in depolarized membranes or use-dependence increases their selectivity. SN dopaminergic neurons, and other neurons at risk in PD that have been studied, are spontaneously active and reside primarily at membrane potentials where DHPs and cp-PYTs preferentially bind to the DHP site.

A shared binding site

Our studies support the conclusion that DHPs and cp-PYT share a common binding pocket. The first piece of evidence supporting this conclusion is that inhibition of Ca_v1.3 channels by the DHP isradipine and by cp-PYT is dependent upon threonine at 1081, a residue found in the DHP binding pocket common to Ca_v1.2 and Ca_v1.3 α_1 subunits (Fig. 2a–d).^{18, 19, 20} Substitution of threonine at 1081 with tyrosine disrupted the ability of isradipine and cp-PYT to inhibit evoked currents. An additional piece of evidence for a shared binding site is that equilibrium membrane binding experiments revealed that isradipine displaced binding of a tritiated PYT (D11), and tritiated D11 binding was displaced by cp-PYT (Fig. 1e–h).

Elucidation of the important residues in the DHP site that determined selective binding of cp-PYT to the Ca_v1.3 α_1 subunit came from our homology models constructed from the Ca_v1.1 structure.^{23, 40} Examination of the region near the conserved threonine residue (1081 in Ca_v1.3 and 1066 in Ca_v1.2) revealed minor structural differences between Ca_v1.2 and Ca_v1.3 subunits (Fig. 2a,b). Docking simulations revealed that in the Ca_v1.3 α_1 subunit, the 3-chlorophenethyl moiety of cp-PYT interacts with methionine-1078, but isradipine does not. Sequence alignment showed that methionine-1078 in the Ca_v1.3 α_1 subunit is not conserved in the Ca_v1.2 α_1 -subunit, but is replaced by a valine (at 1063). Another structural difference seen in the homology models is that the valine at 1198 in the IV transmembrane

domain of the Ca_v1.3 subunit is replaced by isoleucine at 1183 in the Ca_v1.2 subunit. In the homology model, isradipine could be docked into the DHP binding pocket regardless of which amino acid was present. However, valine at 1063 in Ca_v1.2 created significant steric hinderance to cp-PYT binding but isoleucine 1183 caused only slight repulsion (Supporting Information Fig. S3). To test the homology modeling results, site-directed mutagenesis was performed. Mutation of methionine 1078 to valine in Ca_v1.3, thereby giving it Ca_v1.2 character, had no effect on the inhibition produced by isradipine, but dramatically attenuated the effects of cp-PYT on Ca_v1.3 channel currents (Fig. 2e) – outcomes remarkably predicted by the homology model. In contrast, mutation of valine at 1198 in Ca_v1.3 to isoleucine, again giving it Ca_v1.2 character, had no effect on the ability of isradipine or cp-PYT to inhibit Ca_v1.3 channel currents (Fig. 2e). The homology model also accurately predicted the consequences of the corresponding mutations to the Ca_v1.2 subunit. Specifically, mutation of valine at 1063 to methionine, giving it Ca_v1.3 character, increased the ability of cp-PYT to inhibit evoked currents, whereas mutation of isoleucine at 1183 to valine had no effect on cp-PYT inhibition (Fig. 2f). Neither mutation affected the ability of isradipine to inhibit Ca_v1.2 channel currents (Fig. 2f). Together, these results provide strong support for the proposition that the selective inhibition of Ca_v1.3 channels by cp-PYT depends on its interaction with a single amino acid, methionine at 1078, in the DHP binding pocket.

If this selectivity arises from hydrophobic interactions with methionine 1078, as suggested by our previous structure-activity studies,⁴¹ then selectivity should be a function of the surface area of the side chains of the residue at position 1078 in Ca_v1.3. To test this hypothesis, methionine at 1078 in Ca_v1.3 was mutated to alanine, valine, leucine, isoleucine, or lysine (Fig. 3). Consistent with the homology model predictions, as the surface area of the residue decreased, so did inhibition by cp-PYT. None of these mutations was predicted to affect inhibition by isradipine, and none did. Conversely, mutation of valine at 1063 in the Ca_v1.2 α_1 -subunit to isoleucine, leucine, methionine, or lysine revealed that increasing the surface area at this site increased the ability of cp-PYT to inhibit currents, without affecting the efficacy of isradipine.

Reconciliation with previously reported results

Our results not only provide a map for future drug discovery work, they also provide an explanation for previous work that disputed the inhibitory action of cp-PYT on Ca_v1.3 channels. Ortner et al.¹⁴ claimed that cp-PYT was an agonist, rather than an inhibitor, of Ca_v1.3 channels based on their ability at high micromolar concentrations to slow deactivation of heterologously expressed channels. The cloned α_1 subunit used in their experiments, however, had a mutation at methionine 1078, the residue in native channels now shown to be critical for the binding of cp-PYT to the DHP site (Fig. 2). The ‘agonist’ effect on tail current kinetics is attributable to a low affinity binding site we observed elsewhere on the channel, because it was seen even when the DHP site had been mutated (Fig. 5g). It remains to be determined where this site resides, but it is of marginal interest, as in our experiments with native expression systems, as well as those of others,⁹ there is little evidence that this site significantly mitigates the inhibitory effects of cp-PYT on Ca_v13 channels. What is less readily explained is the apparent failure of cp-PYT to inhibit Ca_v13 channels in adrenal chromaffin cells.¹⁴

In another series of experiments with heterologously expressed Ca_v1.3 channels, Huang et al.¹⁵ found cp-PYT to be a weak inhibitor of Ca_v1.3 channels. As virtually all of these experiments were conducted from negative holding potentials, this result is explained by the voltage-dependent interaction of cp-PYT with the DHP binding site. In our work (Figs. 4 and 5), from a holding potential of –80 mV, which was used in the Huang et al. studies, the inhibition of Ca_v1.3 channel currents by cp-PYT was only modest. But at a more physiologically relevant membrane potential of –50 mV, the inhibition was much more profound. This is precisely the phenomenology expected of a NAM. Lastly, Huang *et al.* described a β subunit dependence of cp-PYT inhibition. Given the robust effect of β subunits on the voltage-dependence of channel gating, most likely, it reflects the effect of voltage on the structure of the α_1 -subunit and on accessibility of the DHP binding site. To test this hypothesis, the relationship between channel activation and cp-PYT inhibition should be systematically explored.

Toward a clinically useful drug

Although cp-PYT discriminates between Ca_v1.3 and Ca_v1.2 channels, it has limitations. The key to moving this effort forward is our discovery that cp-PYT is a voltage-dependent NAM that interacts with the DHP binding site on Ca_v1.3 channels. Thus, drug screening efforts need to reliably manipulate membrane voltage for a structure-activity relationship to be ascertained. Recently developed planar patch clamp workstations should allow this effort to move forward on a reasonable timescale. Among the goals of this effort will be to increase the potency of Ca_v1.3 selective compounds by optimizing the interaction with methionine at 1078, utilizing our homology model for predicted selectivity and efficacy.

CONCLUSIONS

The key findings of our study are that cp-PYT acts as a selective, voltage-dependent, NAM of Ca_v1.3 Ca²⁺ channels by binding to the DHP site on the pore-forming α_1 subunit and that this interaction depends on a Ca_v1.3-specific methionine residue at position 1078. The use of homology models developed based on a Ca_v1.1 structure predicted accurately what should occur upon mutation of various residues in Ca_v1.3 and Ca_v1.2, which would lead to Ca_v1.3 selectivity and that the interaction of cp-PYT with Ca_v1.3 is hydrophobic and sterically controlled. This selective inhibition is shown to be voltage dependent, as expected for a NAM. The mechanism of action of cp-PYT appears to be inhibition of mitochondrial OXPHOS, thereby reducing mitochondrial stress. In addition to effectively inhibiting heterologously-expressed Ca_v1.3 channels, cp-PYT also inhibited natively expressed channels in mouse SN dopaminergic neurons. These studies pave the way for development of Ca_v1.3 channel selective NAMs that could be used in the treatment of a variety of psychomotor disorders, such as Parkinson's disease, autism spectrum disorder, bipolar disorder, and drug abuse, in which opening of these channels contributes to pathogenesis.

MATERIALS AND METHODS

Chemical Synthesis

The synthesis of cp-PYT was previously described.¹³

Synthesis of $^3\text{[H]}$ -D11. To a stirred mixture of NiCl_2 (3 mg, 0.023 mmol) and (3-chlorophenyl)acetonitrile (40 mg, 0.26 mmol) in methanol (3 mL), NaBT_4 (5 mg, 13.1 Ci/mmol) was added portionwise (4 times) over 20 min at room temperature. After gas evolution ceased, NaBH_4 (15 mg) was added to the mixture, which was stirred for an additional 1 h. The mixture was diluted with 10 mL of dichloromethane and mixed with ($\pm$)-endo-norbornyl isocyanate (43 mg, 1.2 equiv) at room temperature. The reaction mixture was stirred for 1 h, quenched with dichloromethane (20 mL) and brine (30 mL). The organic layer was collected, dried with anhydrous MgSO_4 , and concentrated under vacuum to afford a yellow oil. The yellow oil was passed through a 4 g silica gel cartridge using an ethyl acetate and hexane mixture (1:1) to give the desired pure urea (20 mg, 26%). To a solution of the urea (15 mg, 0.05 mmol) in dichloromethane (10 mL), malonyl chloride (10 μL , 0.06 mmol) was added with vigorous stirring. After being stirred for an additional 1 h, the solution was washed with brine, dried over anhydrous MgSO_4 , and concentrated at reduced pressure to a small volume. The resulting mixture was purified using a silica gel cartridge (4 g) to give pure $^3\text{[H]}$ -SKP004D11 (9 mg, 7.7 Ci/mol).

Cell Culture

Rat $\text{Ca}_v1.3 \alpha_1\text{D}$ (GenBank accession number: [AF370010](#)) containing all alternative splice sites, rat $\text{Ca}_v\beta_3$ (GenBank accession number: [M88751](#)), rat $\text{Ca}_v\alpha_2\delta-1$ (GenBank accession number: [286488](#)), and rabbit $\text{Ca}_v1.2 \alpha_1\text{C}$ (GenBank accession number: [P15381](#)) cDNAs were used. The potential differences in drug sensitivity caused by alternative splice isoforms of either $\text{Ca}_v1.2$ or $\text{Ca}_v1.3$ were not evaluated in these studies. All wild-type constructs were provided by Dr. Diane Lipscombe (Brown University) and Dr. Johannes Hell (University of Iowa). General methods for constructs development and stable transfection of $\text{Ca}_v1.3 \alpha_1\text{D}$, $\text{Ca}_v\beta_3$, and $\text{Ca}_v\alpha_2\delta-1$ into HEK-293 cells for FLIPR screens were described in our earlier study¹. *Transient transfected cells:* tsA201 cells were maintained in D-MEM medium supplemented with 10% fetal bovine serum (Life Technologies) without antibiotics. Cells were trypsinized and plated on poly-D-lysine coated coverslips (BD Bioscience) 24 h before transfection. A mixture of $\text{Ca}_v1.3 \alpha_1\text{D}$ or mutated $\text{Ca}_v1.2/3 \alpha_1 \text{C/D}$, $\text{Ca}_v\beta_3$, and $\text{Ca}_v\alpha_2\delta-1$ cDNA (in pcDNA3.1 vectors) at a molar ratio 1:1:1 together with 1/40 (w/w) GFP cDNA (Life Technologies) were transfected into tsA201 cells using Mirus TransIT®-LT1 transfection reagent (Mirus®) according to the manufacturer's protocol. GFP-positively labeled cells were recorded 48 h post-transfection.

Generation of the $\text{Ca}_v1.3/\text{Ca}_v1.2$ mutations

Mutation constructs were generated using the Agilent® Quik®Change II Site-Directed Mutagenesis Kit (catalog#: 200524-5). Primer design and PCR was performed according to the standard procedures detailed in the instruction manual. In short, rat $\alpha_1\text{D}$ and rabbit $\alpha_1\text{C}$ genes were mutated to desired constructs using primers detailed in Table S1, S2. PAGE purified primers were made by IDT. Following the digestion with *Dpn* I, the PCR mixture was used to transform XL1-Blue supercompetent cells and the sample was plated onto LB + ampicillin (100 $\mu\text{g/mL}$) plates. The colonies were grown in (5 mL) LB broth containing 100 $\mu\text{g/mL}$ ampicillin and the plasmids were isolated using the Qiagen® QIAprep Spin Miniprep Kit (catalog#: 27104) according the instruction manual. All the mutations were verified by sequencing.

Whole-cell Patch Clamp Electrophysiology

After 48 h of incubation at 37 °C on poly-D-lysine treated coverslips, transiently transfected cells underwent whole-cell patch-clamp electrophysiology. The external solution contained the following (in mM): 140 NaCl, 2 KCl, 2 MgCl₂, 1 CaCl₂, 15 HEPES, and 10 dextrose at pH 7.4 and an osmolarity of ~305 mOsm/L. Barium currents were resolved using a solution with the following (in mM): 140 NaCl, 2 KCl, 2 MgCl₂, 10 BaCl₂, 10 HEPES, and 10 dextrose at pH 7.4 and an osmolarity of ~310 mOsm/L. The test compound stock solutions in DMSO (10 mM or just DMSO) were diluted in the barium recording solution to the desired saturating concentration, which was perfused (2 mL/min) into the recording chamber while measuring the evoked barium currents. Barium currents were measured from whole-cell voltage patch-clamp recordings using the Pulse 8.4 software data acquisition system (HEKA, Germany). Signals were low-pass filtered at 1 kHz, digitized (sampled) at 10 kHz and were amplified with an Axopatch 200B patch-clamp amplifier (Axon Instruments). Barium currents were evoked by a depolarizing voltage step from a holding potential of -80 mV to 0 mV for 100 msec with a frequency of 0.05 Hz at room temperature (22 °C -25 °C). Patch pipettes were pulled from thin-wall borosilicate glass (Sutter Instruments) coated with dental wax and maintained at a resistance of approximately 3–4 mΩ. Internal pipette solutions contained the following (in mM): 180 NMG (N-methyl-D-glucosamine), 40 HEPES, 4 MgCl₂, 12 phosphocreatine, 0.1 leupeptin, 2 Na₂ATP, 0.5 Na₃GTP, 5 BAPTA, pH 7.2–7.3 neutralized by addition of phosphoric acid and an osmolarity of ~280 mOsm/L. Electrophysiological signals were analyzed using Clampfit® 9.2 (Axon Instruments) and IgorPro6® software.

Binding experiments

Ca_v1.3 membranes from stably expressing HEK-293 cells (~1 X 10⁷ cells/harvest) were washed twice with HEPES buffer (40 mM HEPES, 100 mM NaCl, pH 8.0) and then removed from culture dishes. After harvesting the cells by low speed centrifugation (4,000 X g for 15 min) and resuspension in HEPES buffer with NaCl, cells were lysed with a hand-held Potter homogenizer on ice, and membranes were harvested by high speed centrifugation (100,000 X g for 30 min at 4 °C). These homogenization and centrifugation steps were repeated two additional times. A BSA assay was conducted to determine the protein concentration per sample, and for all of the experiments carried out in this study, 0.4 mg/mL/reaction was used. Reaction mixtures for binding experiments consisted of the following (which were added in this order to a total volume of 1 mL): HEPES buffer, membrane fractions, cold (non-tritiated) compound, and hot (tritiated) compound. The reactions were stopped after 90 min incubation at room temperature and were washed, vacuum filtered, and collected on Whatman GF/C filter paper. After incubation in scintillation fluid for 1 h at room temperature, the amount of bound tritium was determined by scintillation counting. Nonspecific binding of SKP004D11 was determined by displacement of tritiated SKP004D11 by a saturating concentration of 'cold' compound. The *K_d* of SKP004D11 for Ca_v1.3 LTCCs was derived from a least-squares fit of a Langmuir isotherm (Fig. 1f) and the negative-inverse of the slope from a Scatchard plot (Fig. 1g). Displacement data were fit with a Langmuir isotherm using a least-square method and the *K_i* estimated using the Cheng-Prusoff equation. For all binding experiments, the ³H-

SKP004D11 concentration was 3.0 nM and membrane protein concentrations for stably expressed Cav1.3 LTCCs in HEK-293 cells were between 0.1 and 0.4 mg/mL.

Generation of Cav1.3/Cav1.2 homology models and molecular docking

The structural models of $\alpha 1$ subunit of Cav1.2 and Cav1.3 were built using the recently reported Cryo-EM structure of rabbit Cav1.1 (3.6 Å) (PDB code: 5GJW) as a structural template. The missing or overlapping residues, charge, and protonation state of Cav1.1 were fixed using the Protein Preparation Wizard module in Schrödinger 11.9. The sequences of four domains (I-IV) that have voltage sensing segments (S1-S4), central pore forming segments (S5-S5'), and P-loop of Cav1.2 (CAC1C_HUMAN) as well as Cav1.3 (CAC1D_HUMAN) were extracted and aligned with the Cav1.1 template using a previously described method²³. The sequence alignment was also checked using Clustal omega multiple sequence alignment program. In this model, long extracellular loops that are far from the DHP binding site are excluded because of a lack of structural information. The sequence similarity between Cav1.1 and Cav1.3 is 67% after exclusion of long extracellular loops. The mapping of protein sequences in 3-D space was achieved using the Homology Modeling module, and the energy minimization of produced structures was performed using the Prime module (OPLS3e force field) in Schrödinger software. Docking of ligands with produced proteins was performed with the Induced Fit Docking module in the Schrödinger software package. The protonation, charges, and alternative overlapping atoms were refined using the Protein Preparation Wizard module in Schrödinger software.

Statistics

To avoid errors associated with deviations from normality in the sampling distributions, box plots were used to present data summaries. In box plots, the median is shown as the central bar of the box and the upper and lower interquartiles are presented at the edges of the box; the line from the top and bottom of the box show the distribution limits, excluding outliers (defined as points that are greater than 1.5X the interquartile range from the median). Hypothesis testing relied on the non-parametric, Mann-Whitney U test of significance unless otherwise stated. Differences were considered statistically significant if $p < 0.05$. Control and experimental groups were interleaved where possible.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

PD	Parkinson's disease
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ASD	Autism spectrum disorder
DHP	dihydropyridine
NAM	Negative allosteric modulator
PYT	Pyrimidine-2,4,6-trione
cp-PYT	1-(3-Chlorophenethyl)-3-cyclopentylpyrimidine-2,4,6-trione
SN	Substantia nigra
OXPHOS	Oxidative phosphorylation
ROS	Reactive oxygen species

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Figure 1. cp-PYT and isradipine are sensitive to the Ca_v1.3-T1081Y mutation.

a. Schematic diagram of the α_1 -subunit for neuronal L-type calcium channels. The third domain is highlighted, showing the amino acids' sequence of the S5 segment that confers 1,4-dihydropyridine (DHP) sensitivity: Ca_v1.3-T1081 and Ca_v1.2-T1066. **b.** T1081 on the Ca_v1.3 subunit was mutated to Y, which should render the channels insensitive to DHPs. **c.** Both 5 μ M isradipine (blue) and 100 μ M cp-PYT (red) failed to significantly inhibit currents in Ca_v1.3-T1081Y channels, suggesting a common binding site. **d.** Box plot summarizing the percent inhibition observed after application of either 5 μ M isradipine (blue) or 100 μ M

cp-PYT (*red*) to wild-type Cav1.3 (isradipine: n = 5, median = 90%; cp-PYT: n = 7, 60%) and Cav1.3-T1081Y (isradipine: n = 5, median = 1%; cp-PYT: n = 5, median = 2%) Ca²⁺ channels. Both the isradipine and cp-PYT effects were statistically significant (Mann-Whitney Rank Sum Test, **p < 0.001). **e.** SKP004D11, a potent and Cav1.3 selective inhibitor was tritiated in order to label the cp-PYT binding site on Cav1.3 L-type calcium channels. The specific binding of ³H₂-SKP004D11 was determined with a displacement assay using non-labeled SKP004D11 (n = 6, median specific binding = 59%). **f.** ³H₂-SKP004D11 specific binding was displaced from Cav1.3 LTCCs in dissociated HEK293 membranes in a dose-dependent manner that was fit with a Langmuir isotherm (K_i = ~60 nM; n = 4; means are plotted). **g.** Scatchard plot of the ³H₂-SKP004D11 displacement data from Cav1.3 channels in dissociated membrane preparations (*red*; mean values from four independent experiments). The K_d of SKP004D11 for Cav1.3 LTCCs was derived from the negative-inverse of the slope from the Scatchard plot (n = 4, median = 40 nM). **h.** Isradipine displaced ³H₂-SKP004D11 (K_i = 68 nM). This was determined by a least-squares fit of a Langmuir isotherm and employment of the Cheng-Prusoff equation. For all binding experiments the ³H₂-SKP004D11 concentration was 3.0 nM and membrane protein concentrations for stably expressed Cav1.3 LTCCs in HEK-293 cells were between 0.1 and 0.4 mg/mL (see Experimental Section: Binding Experiments). **i.** Isradipine binding to HEK293 membranes is dependent on the presence of Cav1.3 channel protein.

Figure 2. Homology model of Cav1.3 and docking of cp-PYT reveals that cp-PYT uniquely engages Met1078 in Cav1.3-LTCCs.

Proposed docking poses of isradipine (**a**) and cp-PYT (**b**) in the Cav1.3 structural model. Isradipine is represented in purple and cp-PYT is represented in orange, while Cav1.3 is represented by green ribbon diagrams. Key binding site residues and interactions with ligands are shown with dark green sticks and with yellow dash lines. Sequence numbers are based on rat Cav1.3 and Cav1.2 subunits. **c**. Schematic diagram of the Cav1 α_1 -subunit. The consensus amino acid sequence of the S5 segment of the third (blue) and the S6 segment of the fourth (green) transmembrane domains of nLTCCs are shown. The docking model predicts that methionine-1078 and valine-1198 (red) of the Cav1.3 α_1 -subunit underlie cp-PYT selective inhibition. **d**. Voltage-clamp currents evoked in HEK293 cells

expressing Ca_v1.3 (left) or Ca_v1.2 (right) channels; Ca_v1.3 channels have a pore-forming α_1 subunit with an M1078V mutation, whereas Ca_v1.2 channels had a pore-forming α_1 subunit with an V1063M mutation. Current records are shown before and after bath application of cp-PYT (100 μ M). Voltage protocol is shown at the bottom. **e.** Population data of the inhibition observed by application of either 100 μ M cp-PYT (*left*) or 5 μ M isradipine (*right*) to Ca_v1.3 (*black*, cp-PYT: n = 6, 60%, isradipine: n = 5, 90%), Ca_v1.3-M1078V (*blue*, cp-PYT: n = 6, 4%, isradipine: n = 5, 78%), and Ca_v1.3-V1198I (*green*, cp-PYT: n = 6, 45%, isradipine: n = 6, 94%) L-type calcium channels (LTCCs). The inhibition of Ca_v1.3 as compared to Ca_v1.3-M1078V LTCCs by application of 100 μ M cp-PYT was deemed significant by the Mann-Whitney Rank Sum Test (**p < 0.001). **f.** Population data of the inhibition observed by application of either 100 μ M cp-PYT (*left*) or 5 μ M isradipine (*right*) to Ca_v1.2 (*black*, cp-PYT: n = 8, 21%, isradipine: n = 6, 78%), Ca_v1.2-V1063M (*blue*, cp-PYT: n = 6, 47%, isradipine: n = 6, 92%), Ca_v1.2-I1183V (*green*, cp-PYT: n = 8, 12%, isradipine: n = 8, 95%), and Ca_v1.2-V1063M+I1183V (*gray*, cp-PYT: n = 10, 32%, isradipine: n = 10, 93%) L-type calcium channels. The inhibition of Ca_v1.2 as compared to Ca_v1.2-V1063M and Ca_v1.2-V1063M+I1183V by application of 100 μ M cp-PYT was deemed significant by the Mann-Whitney Rank Sum Test (*p < 0.05).

Figure 3. Modulating the steric demand of a single amino acid residue directs pyrimidine-2,4,6-trione sensitivity.

a. Schematic drawing of amino acids placed into the α_1 -subunit of $\text{Ca}_v1.3$ at position 1078 or $\text{Ca}_v1.2$ at position 1063. Amino acids are shown by size of accessible surface area. The native amino acid at these aligned positions is methionine for $\text{Ca}_v1.3$ (black) and valine for $\text{Ca}_v1.2$ (red). **b.** Resultant averaged current traces of $\text{Ca}_v1.3$ L-type calcium channels, evoked by a -80 mV to 0 mV voltage step for 100 msec, before (gray) and after 100 μM cp-PYT (blue) application. Each pairing of traces represents mutations at $\text{Ca}_v1.3$ –1078 that express amino acids of descending size of accessible amino acid surface area. **c.** Resultant averaged current traces of $\text{Ca}_v1.2$ L-type calcium channels, evoked by a -80 mV to 0 mV voltage step for 100 msec, before (gray) and after (blue) 100 μM cp-PYT application. Each pairing of traces represents mutations at $\text{Ca}_v1.2$ –1063 that express amino acids of ascending size of accessible amino acid surface area. All horizontal scale bars represent 50 msec. **d.** Population data of the inhibition observed by 100 μM cp-PYT application on L-type calcium channels with differing sizes of accessible amino acid surface area at either $\text{Ca}_v1.3$ –1078 (black symbols): $\text{Ca}_v1.3$ –M1078K ($n = 5$, median = 58%), $\text{Ca}_v1.3$ –M1078I ($n = 6$, median = 37%), $\text{Ca}_v1.3$ –M1078L ($n = 5$, median = 31%), $\text{Ca}_v1.3$ –M1078V ($n = 8$, median = 4%), and $\text{Ca}_v1.3$ –M1078A ($n = 4$, median = 17%); or at $\text{Ca}_v1.2$ –1063 (red symbols): $\text{Ca}_v1.2$ –V1063I ($n = 5$, median = 48%), $\text{Ca}_v1.2$ –V1063L ($n = 4$, median = 35%), $\text{Ca}_v1.2$ –V1063M ($n = 6$, median = 47%), and $\text{Ca}_v1.2$ –V1063K ($n = 4$, median = 40%) relative to the inhibition of 100 μM cp-PYT on wild-type $\text{Ca}_v1.3$ (black symbols) ($n = 5$, median = 60%) or $\text{Ca}_v1.2$ (red symbols)

(n = 6, median = 21%) LTCCs. Open black circles (Ca_v1.3) or red circles (Ca_v1.2) represent individual experiments, while filled circles represent median responses. Linear regression analysis of the median cp-PYT responses led to a linear relationship of the % inhibition and size of accessible side chain (n = 11, $r^2 = 0.57$). **e.** Population data of the inhibition observed by 5 μ M isradipine for channels with differing sizes of accessible amino acid surface area at either Ca_v1.3–1078 (black symbols): Ca_v1.3-M1078K (n = 4, median = 88%), Ca_v1.3-M1078I (n = 5, median = 85%), Ca_v1.3-M1078L (n = 6, median = 69%), Ca_v1.3-M1078V (n = 6, median = 78%), and Ca_v1.3-M1078A (n = 4, median = 84%); or at Ca_v1.2–1063 (red): Ca_v1.2-V1063I (n = 5, median = 91%), Ca_v1.2-V1063L (n = 6, median = 95%), Ca_v1.2-V1063M (n = 6, median = 92%), and Ca_v1.2-V1063K (n = 4, median = 72%) relative to the inhibition of 5 μ M isradipine on wild-type Ca_v1.3 (black symbols) (n = 7, median = 90%) or Ca_v1.2 (red symbols) (n = 5, median = 78%) LTCCs. Open circles black (Ca_v1.3) or red circles (Ca_v1.2) represent individual experiments, while filled circles represent medians. Linear regression analysis of the median isradipine responses did not result in a linear relationship of the % inhibition and size of accessible side chain (n = 11, $r^2 = 0.04$).

Figure 4. cp-PYT inhibition of Ca_v1.3 channel currents is voltage-dependent.

a. Exemplary current traces evoked in HEK293 cells expressing Ca_v1.3 channels before (control, black) and after application of isradipine (100 nM, blue) from either a holding potential (V_H) = -80 mV (left) or V_H = -50 mV (right). The test step voltage was 0 mV. **b, c.** Examples of current traces before (black) and after cp-PYT (10 μM, red) application from either V_H = -80 mV (**b**, left) or V_H = -50 mV (**c**, left) (test step was to 0 mV). Time course of cp-PYT and Cd²⁺ inhibition of Ca_v1.3 peak currents from either V_H = -80 mV (**b**, right) or V_H = -50 mV (**c**, right). **d.** Box plot summary of percentage inhibition of Ca_v1.3 peak currents by isradipine and cp-PYT at either V_H = -80 mV or -50 mV (test step to 0 mV). Isradipine (V_H: -50 mV, n = 6, median = 92% vs. V_H: -80 mV, n = 5, median = 43%; Mann-Whitney test, P = 0.0043). cp-PYT (V_H: -50 mV, n = 6, median = 46% vs. V_H: -80 mV, n = 5, median = 26%; Mann-Whitney test, P = 0.0058).

Figure 5. High micromolar concentrations of cp-PYT slowed tail currents independent of the effect on peak current.

a. $\text{Ca}_v1.3$ channel currents evoked in HEK293 cells by a voltage step to 0 mV (holding at -80 mV) before and after application of cp-PYT ($10 \mu\text{M}$). **b.** Semilog plots of absolute tail currents from **a**. **c.** Summary of the principal tail current time constant before (black) and after (red) compound application. There were no changes ($n = 4$, paired t-test, $p = 0.86$). **d.** Exemplary current traces evoked by the same protocol before and after application of cp-PYT at high concentration ($50 \mu\text{M}$). **e.** Semilog plot of absolute tail currents from $\text{Ca}_v1.3$ channels before (black) and after (red) cp-PYT ($50 \mu\text{M}$) application (**e**) cp-PYT concentration. **f.** Plot of the principal tail time constants before and after cp-PYT application. Note the slowing of the tail current kinetics ($n = 3$, paired t-test, $p = 0.04$). **g.** Semilog plot of absolute tail currents from $\text{Ca}_v1.3$ -M1078V mutant channels from before (black) and after (red) cp-PYT ($50 \mu\text{M}$) application. **h.** Plot of the principal tail time constant before and after cp-PYT application ($n = 4$, $*p < 0.05$, Mann-Whitney).

Figure 6. cp-PYT inhibits $\text{Ca}_v1.3$ calcium channels in adult SN dopaminergic (DA) neurons from wild-type mice.

a. Left, TH-positive SN DA neuron (scale bar, 10 μM). Right, cell-attached firing (2–4 Hz) from a typical TH-positive, dissociated SN DA neuron. **b.** Voltage clamp recordings of an adult (P25) SN dopaminergic neuron from an adult WT mouse showing inhibition by cp-PYT (5 μM) (gray) and (50 μM) (red) application. **c.** Voltage clamp recordings of an adult (P25) SN dopaminergic neuron from an adult $\text{Ca}_v1.3$ -KO mouse showing a lack of inhibition by cp-PYT (50 μM) application (red). **d.** Box-plot summarizing a dose-dependent effect of cp-PYT (gray, 5 μM) and (red, 50 μM) application on the maximum calcium permeability of SN dopaminergic neurons from adult WT mice as compared to the maximum calcium permeability of SN DA neurons from a $\text{Ca}_v1.3$ -KO mouse (black, $n = 6$, $p < 0.001$, Mann-Whitney for 50 μM). **e.** Left, Drawing of an SN dopaminergic neuron derived from a z-stack obtained with two photon laser scanning microscopy following filling of the cell with Alexa568 (20 μM) and Fluo-4 (200 μM) through a whole-cell recording glass pipette. Calcium imaging was performed along the dendrite, ~100 pm from the soma (location indicated by arrow). Somatic voltage recordings before (left, black) and after (right, red) application of cp-PYT (20 μM). 2PLSM measurements of Fluo-4 fluorescence

normalized by the baseline Fluo-4 fluorescence (F_0) before (black) and after (red) cp-PYT application. **f.** Comparison of single calcium transients revealed a marked reduction in amplitude following cp-PYT administration; hashed line denotes the point at which the spike occurred. **g.** Summary showing that cp-PYT significantly reduced the amplitude of spike-associated dendritic Ca^{2+} transients ($p < 0.01$; $n = 6$, Mann-Whitney). **h.** Top, schematic of the hypothesized signaling between $\text{Ca}_v1.3$ channels and mitochondria. Bottom, a high magnification image of a SN dopaminergic neuron from a transgenic TH-mito-roGFP mouse showing cytoplasmic, but not nuclear, labeling. **i.** Mito-roGFP measurements from a SN dopaminergic neuron revealed a high basal oxidation (left, black trace). Isradipine (right, black trace) and cp-PYT (right, red trace) treatment diminished mitochondrial oxidation. **j.** Box-plots summarizing mean redox measurements in control (black, $n = 9$) and following isradipine treatment (black, $n = 8$) or cp-PYT treatment (red, $n = 6$); mitochondrial oxidant stress was significantly diminished by both isradipine and cp-PYT treatment ($*p < 0.05$, Mann-Whitney).