Pantothenate kinase (PANK) is a metabolic enzyme that regulates cellular coenzyme A (CoA) levels. There are three human PANK genes, and inactivating mutations in PANK2 lead to pantothenate kinase associated neurodegeneration (PKAN). Here we performed a library screen followed by chemical optimization to produce PZ-2891, an allosteric PANK activator that crosses the blood brain barrier. PZ-2891 occupies the pantothenate pocket and engages the dimer interface to form a PANK•ATP•Mg^{2+}•PZ-2891 complex. The binding of PZ-2891 to one protomer locks the opposite protomer in a catalytically active conformation that is refractory to acetyl-CoA inhibition. Oral administration of PZ-2891 increases CoA levels in mouse liver and brain. A knockout mouse model of brain CoA deficiency exhibited weight loss, severe locomotor impairment and early death. Knockout mice on PZ-2891 therapy gain weight, and have improved locomotor activity and life span establishing pantazines as novel therapeutics for the treatment of PKAN.
A rare, life-threatening neurological disorder known as pantetheine kinase-associated neurodegeneration (PANK) arises from mutations in the human PANK2 gene leading to a prominent extrapyramidal movement disorder and a characteristic deposition of iron in the basal ganglia. Pantetheine kinase (PANK, EC 2.7.1.33) is the first and rate-controlling step in the only pathway for coenzyme A (CoA) biosynthesis. CoA is a major acyl group carrier in biology and participates as a key cofactor and regulator of intermediary metabolism. Three genes express four closely-related mammalian isoforms: PANK1α, PANK1β, PANK2, and PANK3. The isoform expression level and potent feedback inhibition by acyl-CoAs control the intracellular CoA content. The physiological evaluation of PANK knockout mice and mice treated with a PANK inhibitor establish a key role for the intracellular CoA concentration in supporting oxidative metabolism, ketone utilization and glucose homeostasis. PKAN pathology is thought to arise from neuronal CoA deficiency. This view is strengthened by the recent discovery that mutations in CoA synthase cause a similar neurodegenerative disease. The PANK2 gene is abundant in human neuronal tissues and the majority of the mutations associated with PKAN result in the expression of truncated or mutant PANK2 proteins with little or no catalytic activity. Human PANK2 is localized to the mitochondrial intermembrane space but a mouse PanK2 mitochondrial targeting sequence has not been identified, and others report a cytosolic localization. Mouse PanK2 was reported to be mitochondrial, but a mouse PanK2 mitochondrial targeting sequence has not been identified. Human PANK2 is localized to the mitochondrial intermembrane space.

There are no disease-modifying treatments for PKAN, and therapeutics are desperately needed. One approach is to use compounds with physicochemical properties suitable for biophysical quantitatively compare the molecules, and ranks them based on physicochemical properties suitable for biophysical characterization. These efforts prioritized a piperazine urea hit, PZ-2789 (Fig. 1a), which was subjected to a hit-to-lead optimization using LipE as the primary driving metric to create a compound series called pantazines (Fig. 1a). PANK activation in cells (see below) requires a high-affinity pantazine, and a PANK inhibition assay without acetyl-CoA was used to rank the pantazines. The structure–activity relationships revealed the importance of a small branched alkyl group at R₁, a carbonyl (H-bond acceptor) next to the piperidine ring, and an electron

Here, we report the development of a drug capable of allosterically activating the alternate PANK isoforms as a potential PKAN therapeutic. The lead pantazine, PZ-2891, arose from the LipE-guided chemical optimization of a hit from a high-throughput screen designed to identify inhibitors and activators of PANK. Due to the high cooperativity of the PANK dimer the binding of PZ-2891 to one protomer locks the opposite protomer in a constitutively active state that is refractory to feedback inhibition by acetyl-CoA. PZ-2891 crosses the blood brain barrier to elevate brain CoA. A mouse model of CoA deficiency employing the neuron-selective deletion of PANK1 and PANK2 genes exhibits weight loss, severely impaired locomotor activity, and early death. PZ-2891-treated knockout mice gain weight, and have improved locomotor activity and life span. These data suggest pantazines as a novel approach to treating PKAN.

Results
LipE-guided optimization identifies PZ-2891 as a PANK modulator. Prior attempts at hit-to-lead optimization of hits from a large high throughput screen for modulators of PANK2 failed to generate suitable chemical leads due to flat structure-activity relationships and poor solubility. To address these shortcomings, we reevaluated the hit list using the alternative approach of filtering for compounds with both lead-like molecular weight (<350) and lipophilic ligand efficiency (LipE > 2). LipE (pIC50 − cLogP) blends both potency and lipophilicity to quantitatively compare the molecules, and ranks them based on physicochemical properties suitable for biophysical characterization. These efforts prioritized a piperazine urea hit, PZ-2789 (Fig. 1a), which was subjected to a hit-to-lead optimization using LipE as the primary driving metric to create a compound series called pantazines (Fig. 1a). PANK activation in cells (see below) requires a high-affinity pantazine, and a PANK inhibition assay without acetyl-CoA was used to rank the pantazines. The structure–activity relationships revealed the importance of a small branched alkyl group at R₁, a carbonyl (H-bond acceptor) next to the piperidine ring, and an electron

Fig. 1 Chemical progression of pantazines. a Lipophilic ligand efficiency (LipE = pIC50 − cLogP) guided optimization of the pantazine series from the initial hit (PZ-2789) to PZ-2891. b Summary of the structure-activity relationships of >60 compounds synthesized during the optimization of the pantazine scaffold. c Chemical structure of an inactive pantazine, PZ-3067, which is used as a negative control in cellular assays.
Pantazines bind to the PANK3-ATP\(\cdot\)Mg\(^{2+}\) complex. The lead pantazine, PZ-2891, inhibited PANK3 with nM affinity, whereas the inactive PZ-3067 had no effect (Fig. 2a). PZ-2891 inhibited all three human and mouse pantothenate kinase isoforms (Supplementary Fig. 1). We used a less potent pantazine, PZ-2724 (IC\(_{50}\) = 1.1 \(\mu\)M) to determine how pantazines interrupted the ordered kinetic mechanism of PANK3 (Supplementary Fig. 2). Kinetic analysis of PANK3 with respect to ATP showed that PZ-2724 was an uncompetitive inhibitor resulting in a decreased V\(_{\text{max}}\) and ATP Km consistent with the pantazines binding to the PANK3-ATP\(\cdot\)Mg\(^{2+}\) complex. Pantazine inhibition with respect to pantothenate was noncompetitive. The conclusion that pantazines bind tightly to the PANK3 pantothenate was noncompetitive and be inhibited by, acetyl-CoA. Within the cell, the PANK3 dimer exists in one of two distinct conformations: the inactive conformation stabilized by the binding of acetyl-CoA and the active conformation stabilized by the binding of ATP\(\cdot\)Mg\(^{2+}\). In the normal catalytic cycle, ATP cooperatively binds to the PANK dimer switching both protomers to the active, closed conformation. Pantothenate binds and catalysis proceeds through the three, structurally characterized intermediates, and both of the active sites exist. The phosphopantothenate product is rapidly converted to CoA by the biosynthetic pathway. Acetyl-CoA is a feedback inhibitor of PANK, and cellular acetyl-CoA levels rise until almost all of the PANK exists in the acetyl-CoA-bound, inactive protein conformation.

Sub-saturating PZ-2891 concentrations interrupt this normal catalytic cycle and feedback regulatory mechanism. In the in vitro biochemical assays to optimize the pantazine series, acetyl-CoA was absent and the presence of ATP (>1 mM) ensured that PANK3 existed only in the active conformation. Under these conditions, PZ-2891 only acts as an inhibitor by titrating the active sites (Fig. 3a). However, the presence of acetyl-CoA in cells means that upon completion of the catalytic cycle PANK3 is available to bind acetyl-CoA and switch to the inactive conformation. When PZ-2891 is bound to only one protomer, the opposite protomer remains capable of catalysis. However, at the end of the pantazine-dependent catalytic cycle, PANK3 remains locked in its active conformation by the pantazine preventing binding of the acetyl-CoA inhibitor and allowing another cycle of catalysis to proceed (Fig. 3a). This effect renders PANK3 dimers with PZ-2891 bound to only one protomer refractory to feedback inhibition by acetyl-CoA (Fig. 3a).

The unusual effect of PZ-2891 acting as both an orthosteric inhibitor and an allosteric activator of PANK3 activity in the presence of acetyl-CoA was tested in biochemical assays designed to mimic the mixture of ligands present in cells. The assays contained either PANK3 or PANK3 plus PZ-2891 at a concentration of drug that partially inhibited (25%) the total PANK3 activity. In the absence of PZ-2891, PANK3 activity was extinguished in a concentration-dependent manner by acetyl-CoA as is normally observed. However, in the presence of PZ-2891 approximately half of the PANK3 activity.
Fig. 2 PZ-2891 binds to the PANK3•ATP•Mg2+ complex with nM affinity. a PZ-2891 (n = 10) (filled circles) and PZ-3067 (open circles) (n = 2) inhibition of PANK3. The data were fit to the Morrison equation and 1.3 ± 0.2 nM was the consensus PZ-2891 IC50 from five experiments using different batches of purified enzyme. A representative data set is shown. b Thermal stabilization of PANK3 by 8 mM ATP, 8 μM PZ-2891 or 2 mM ATP plus 2 μM PZ-2891. Control (red line), PZ-2891 (black line), ATP (green line), and ATP plus PZ-2891 (blue line). The peaks of the first derivative plots of the thermal denaturation curves identify the temperatures at which 50% of the protein is unfolded. The shifts in the thermal denaturation temperatures were calculated from experiments performed in triplicate, and the averages rounded to the nearest degree. c Isolation of the PANK3•[3H]ATP•Mg2+•PZ-2891 complex by gel filtration chromatography. Black trace, PANK3 protein elution profile (A280); Red trace, [3H]ATP elution profile in the presence of PANK3 + PZ-2891; Green trace (on baseline), [3H]ATP elution profile with PANK3 alone. One example of duplicate experiments is shown. d Surface plasmon resonance analysis of PZ-2891 binding to PANK3 in the presence of 1 mM ATP•Mg2+. The data (black lines) were fit to a 1:1 binding model (orange lines). The association (k_a), dissociation (k_d), equilibrium (K_D) constants were calculated as 2.37 × 10^6 M^-1 s^-1, 4.82 × 10^-4 s^-1 and 0.203 nM respectively. The residence time (1/k_d) of PZ-2891 was 34 min. Data are the average of two experiments in duplicate. e Overview of the PANK3 dimer illustrating that PZ-2891 binds across the dimer interface. The two PANK3 protomers are colored cyan and gold, PZ-2891 is purple and AMPPNP is green. f Close-up view of PZ-2891 bound across the dimer interface of the PANK3•AMPPNP•Mg2+ complex illustrating the key hydrogen bonding interactions (dotted red lines) with both PANK3 protomers. g Structure of the PANK3•AMPPNP•Mg2+•pantothenate (Pan) complex (PDB ID: 5KPR) with the pantothenate hydroxyl rotated into the catalytically active position.
remained in the presence of PZ-2891 was refractory to acetyl-CoA inhibition (Fig. 3b). These data directly support the existence of the drug-induced catalytic cycle that prevents the enzyme from interacting with acetyl-CoA. A second experiment to model the cellular environment employed PANK3 whose activity was repressed >95% by the presence of acetyl-CoA (Fig. 3c). Under these conditions, an increase in pantothenate from 45 μM to 90 μM had little impact on the rate because PANK3 existed in a conformation unable to bind the substrate. The addition of PZ-2891 activated PANK3, and increasing the pantothenate concentration to twice its Km further accelerated PANK3 activity (Fig. 3c). These data are consistent with pantazines not affecting the pantothenate Km (Supplementary Fig. 2c), and predict that pantothenate concentrations above the Km for PANK would provide the highest level of pantazine-dependent PANK activity in the presence of acetyl-CoA. These data predict that pantazine-treated cells would have elevated CoA and reduced pantothenate due to the activation of PANK. C3A cells were labeled with [3H]pantothenate in the presence and absence of 10 μM PZ-2891 and the CoA and pantothenate levels

**Fig. 3** Activation of PanK by PZ-2891. **a** Kinetic model illustrating how PZ-2891 binding activates PANK3. PANK3 is a highly-cooperative enzyme where both active sites exist in either the active or inactive conformation. The inactive ‘open’ conformation (red rectangles) is stabilized by the binding of acetyl-CoA, and the active ‘closed’ conformation (green or curved shape) is stabilized by ATP binding. In the normal catalytic cycle, PANK3 returns to a ligand-free state that allows it to bind acetyl-CoA and switch to the inactive conformation. PZ-2891 binds tightly to the active, ATP-bound PANK3 conformation. When a fraction of PANK3 is occupied by PZ-2891, the catalytic cycle is operating on only one protomer of the dimer. In this catalytic cycle, the active monomer of PANK3 empties, but remains locked in the active conformation by ATP•Mg2+PZ-2891 binding to the other protomer. The drug-induced catalytic cycle prevents PANK3 from adopting the open, inactive conformation making PANK3 refractory to feedback regulation by acetyl-CoA. Pan pantothenate, P-Pan phosphopantothenate. **b** PZ-2891 renders PANK3 refractory to acetyl-CoA inhibition. PANK3 (1 μg/assay) activity at different concentrations of acetyl-CoA was determined with ATP (1 mM) in the presence (open red circles) and absence (filled circles) of 2.5 μM PZ-2891. The time course assays contained PANK3 (1 μg/assay), ATP (1 mM) and acetyl-CoA (100 μM) such that PANK3 was inhibited about 95% by acetyl-CoA in the absence of pantazine. The addition of 2.5 μM PZ-2891 to the assay increased the PANK3 rate in a pantothenate-dependent manner. Filled black circles are 45 μM pantothenate, filled blue squares are 90 μM pantothenate, red open circles are 45 μM pantothenate plus 2.5 μM PZ-2891, and green open squares are 90 μM pantothenate plus 2.5 μM PZ-2891. Data in **b** and **c** are from two independent experiments that were performed in duplicate. **d** C3A cells were radiolabeled with [3H]pantothenate in the presence (red trace) or absence (black trace) of 10 μM PZ-2891 for 24 h. The cells were extracted, and the labeled metabolites were analyzed by thin-layer chromatography and imaged with a Bioscan detector. Representative chromatograms from two independent experiments in duplicate are shown.
were determined following separation by thin-layer chromatography (Fig. 3d). PZ-2891 treatment increased the amount of intracellular CoA and lowered intracellular pantothenate, thus illustrating the operation of the pantazine-dependent catalytic cycle that functions to elevate CoA in cells (Fig. 3a).

PZ-2891 increases intracellular CoA in cultured cells. As a prelude to examining the pantazine effect in animals, a series of experiments were performed to verify that PZ-2891 targeted PANK3 in cells and to determine if there were any obvious off-target contraindications. A cellular thermal shift assay (CETSA) was used to confirm that PZ-2891 was bound to cellular PANK3. HEK 293 T cells transfected with a plasmid expressing PANK3 were used to increase cellular levels of PANK3 so that the protein could be detected by immunoblotting cell lysates (Fig. 4a). The CETSA assay showed that PZ-2891 stabilized PANK3 by ~7 °C confirming that PZ-2891 bound to PANK3 in intact cells (Fig. 4b). A PZ-2891 CETSA dose-response curve indicated that half of the cellular PANK3 was bound at a concentration of ~9 μM PZ-2891 (Supplementary Fig. 5). PZ-2891 (10 μM) did not impact cell viability as measured by cell growth or the synthesis of DNA, protein and lipid, indicating the absence of significant toxicity toward cultured cells (Supplementary Fig. 6). The propensity of PZ-2891 to interfere with the activity of other cellular processes was evaluated by two enzymatic screens. First, PZ-2891 did not have significant inhibitory activity in a screen of 468 mammalian kinases (Supplementary Fig. 7, Supplementary Dataset 1). The second screen examined the effect of PZ-2891 on 72 cellular proteins known to cause off-target effects in drug discovery (Supplementary Dataset 2). These data show that PZ-2891 selectively targeted PANK in cells and indicated the absence of off-target pharmacological interactions.

The total intracellular CoA levels were determined in a human liver-derived cell line (C3A) treated for 24 h with increasing concentrations of PZ-2891 (Fig. 4c). CoA progressively increased up to 10 μM PZ-2891, whereas 20 and 50 μM were less activating. As expected, cells did not respond to the inactive pantazine, PZ-3067 (Fig. 4c). The activation phenomenon was not due to enhanced transcription of the kinase isoforms (Supplementary Fig. 8). To verify that PZ-2891 action was PANK-dependent, cells were transfected with an expression plasmid to increase the cellular content of PANK3. The addition of 10 μM PZ-2891 to control (empty vector) HEK293T cells also significantly increased the levels of intracellular CoA (Fig. 4d). The plasmid-driven increase in PANK3 expression resulted in higher baseline CoA, and the treatment of PANK3 overexpressing cells with 10 μM PZ-2891 triggered a large increase in intracellular CoA (Fig. 4d). Again, PZ-3067 was inactive. Similar transfection experiments showed that PANK1 expression also raised CoA after a 24 h treatment with 10 μM PZ-2891 (Fig. 4e). There was no PZ-2891-stimulated CoA synthesis in cells expressing the inactive PANK3 (E138A) mutant, consistent with a requirement for catalytically active PANK3 rather than some other function of the protein (Fig. 4e). Along with CoA, the levels of phosphopantetheine and dephospho-CoA and other pathway intermediates were measured (Supplementary Fig. 9). These two pathway intermediates were minor components in both control and PZ-2891-treated cells showing that activation of CoA biosynthesis at the PANK step was not constrained by a secondary control point in the CoA biosynthetic pathway. Mass spectrometry showed that PZ-2891-treated cells had higher acetyl-CoA levels compared to untreated controls (Supplementary Fig. 10), indicating that PZ-2891 effectively prevented feedback inhibition by acetyl-CoA. Once PANK3 is in the active state, the reaction rate is determined by the concentration of pantothenate in relation to the PANK Km (Fig. 3c). A reduction in the pantothenate in the cell culture medium reduced PZ-2891 activation of CoA synthesis, whereas increasing the media concentration potentiated the PZ-2891 effect (Fig. 5a). Pantothenate itself had no effect on CoA content, consistent with PANK as the feedback-regulated, rate-controlling step in the pathway.

PZ-2891 elevates tissue CoA content in mice. The favorable in vitro ADME and in vivo pharmacokinetic properties of PZ-2891 (Supplementary Tables 1, 2) suggested it could be utilized as a proof of principle pantazine to alter CoA levels in a mouse model. Mice were treated with PZ-2891 either with or without a pantothenate supplement included in the oral gavage to determine if pantothenate administration augmented the effect of PZ-2891 in mouse liver as it did in cultured cells. Treatment of mice with the maximum soluble dose of PZ-2891 (30 mg/kg) increased total liver CoA by 50% in animals administered PZ-2891 alone. There was a 100% increase in CoA levels in animals treated with PZ-2891 plus pantothenate (Fig. 5b). We next analyzed target tissue pantothenate levels to determine the pantothenate concentrations in animals maintained on normal chow (25 ppm pantothenate) or animals treated with 200 mg/kg of pantothenate by oral gavage. Pantothenate supplementation increased pantothenate levels in plasma (Fig. 5c), liver (Fig. 5d), forebrain (Fig. 5e) and hindbrain (Fig. 5f). The amounts of pantothenate in liver correlated with the plasma levels, and calculation of the intracellular concentrations in liver showed higher levels of pantothenate than in the plasma in both cases (10.6, and 24.4 μM, respectively). Similarly, pantothenate levels in the hindbrain and forebrain were elevated by pantothenate supplementation; however, the pantothenate levels in brain were significantly higher than in liver. In all circumstances, estimated tissue pantothenate concentrations were below the PANK3 pantothenate Km (45 μM), explaining how the pantothenate supplement potentiates the effect of PZ-2891 on tissue CoA levels.

The potential therapeutic benefit of a treatment for PKAN neurodegeneration rests on its ability to cross the blood–brain barrier and increase CoA levels in the brain. Male and female mice were co-administered increasing concentrations of PZ-2891 to determine if PZ-2891 was capable of elevating CoA in the brain (Fig. 6). The animals received 5 doses of PZ-2891 by oral gavage every 12 h, and tissues were harvested 4 h after the last dose. We observed a dose-dependent increase in total CoA in liver (Fig. 6a, d), forebrain (Fig. 6b, e) and hindbrain (Fig. 6c, f) in PZ-2891-treated male and female animals. PZ-2891 increased CoA in both males and females to a similar extent. In this short treatment regimen, higher PZ-2891 doses were required to maximally increase CoA in the brain compared to the liver. We also measured PZ-2891 levels in tissues from mice treated with PZ-2891 for four weeks, and these data clearly show the presence of drug in liver and brain (Supplementary Fig. 11). To ensure that an active PANK2 was not necessary for the PZ-2891 effect, we treated Pank2−/− mice with PZ-2891 and measured the CoA levels in liver and brain. PZ-2891 effectively increased CoA in the tissues of Pank2−/− knockout mice (Supplementary Fig. 12) illustrating that the expression of PanK2 was not required for pantazine activation of CoA biosynthesis, and that activation of PanK1 and PanK3 were sufficient to raise CoA. PZ-2891 therapy would be maintained for extended periods, therefore we formulated pantothenate-supplemented mouse chow with different concentrations of PZ-2891. Five mice were treated for 1 week with 112 ppm PZ-2891 in the chow, and the elevation in CoA in six tissues were examined (Supplementary Fig. 13). CoA levels were elevated in liver, brain, and to a lesser extent, heart. Muscle CoA levels are the lowest of all tissues, and were not elevated by...
PZ-2891 therapy. A dose-response experiment was conducted by the administration of a series of PZ-2891 doses in the diet (Fig. 6g, i). These experiments showed both liver and brain CoA were significantly elevated by PZ-2891 in a dose-dependent manner. During the one-month treatment there was no difference in the weights or behavior of the treated animals. These data established that long-term PZ-2891 therapy was effective in elevating CoA in the liver and brain of mice.
Pantothenate levels were manipulated by supplementing 8NATURE COMMUNICATIONS |  (2018) 9:4399 | DOI: 10.1038/s41467-018-06703-2 | www.nature.com/naturecommunications

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of PANK2 by activating the two other PANK isoform
the cellular effects of PZ-2891 verify that pantothenate kinase is the only relevant rate-controlling enzyme in mammalian CoA biosynthesis. Although CoA synthase was suggested as a second control point in the CoA biosynthetic pathway, our quantitative identification of pathway intermediates by mass spectrometry shows that significant activation of PANK does not result in the accumulation of other pathway intermediates, such as phosphopantetheine, the CoA synthase substrate. The steady state CoA tissue level is controlled by the relative rates of synthesis and turnover. CoA turnover is thought to be catalyzed by nudix hydrolases, but in cultured cells, nudix hydrolase expression is low and CoA turnover may be slower than in animal tissues. Current medicinal chemistry efforts are directed toward optimizing the pharmaceutical properties of PZ-2891 while maintaining its excellent oral bioavailability, target affinity, and blood brain barrier permeability.

Fig. 6 Activation of CoA synthesis in liver, forebrain and hindbrain of male and female animals treated with PZ-2891. Groups of 5 mice were administered the indicated amounts of PZ-2891 plus 100 mg/kg pantothenate by oral gavage every 12 h for 5 doses. Four hours after the last dose, the tissues were harvested and the CoA levels determined. Control CoA levels (closed circles); PZ-2891 treated CoA levels (open circles). a Male liver. b Male forebrain. c Male hindbrain. d Female liver. e Female forebrain. f Female hindbrain. Groups of 5 mice were maintained on chow fortified with 1000 ppm pantothenate and the indicated levels of PZ-2891. The mice were maintained on the diets for 4 weeks and their tissues were harvested and the CoA levels determined.

- Liver CoA levels.
- Forebrain CoA levels.
- Hindbrain CoA levels. Statistical significance was determined using Student’s t-test calculated with Graph-Pad software and the p values (red) are noted on the figure panels in red. Means ± SEM are plotted.
Fig. 7 PZ-2891 therapy in SynCre+ PANK1,PANK2 neuronal knockout mice. Male and female mice were randomized into the experimental groups when they were genotyped, and the numbers of mice used in each analysis are shown in parenthesis in the figure panels. a Weight monitoring of double knockout mice maintained on the control diet (black circles) or on a diet with PZ-2891 (red circles). The start of treatment and the time of sample collection are indicated with arrows on the figure. b Lifespan of double knockout mice with (red line) or without (black line) PZ-2891 treatment. c Forebrain CoA levels in littermate control (SynCre−) mice (filled circles) compared to SynCre+ Pank1,Pank2 neuronal knockout mice at day 45 with (open squares) or without (filled squares) PZ-2891 therapy. d Hindbrain CoA levels in littermate control (SynCre−) mice (filled circles) compared to SynCre+ Pank1,Pank2 neuronal knockout mice at day 45 with (open squares) or without (filled squares) PZ-2891 therapy. e The percent time mice were moving during the 5 min open field test. Littermate control (SynCre−) mice (untreated, filled circles; treated, open circles) were compared to SynCre+ Pank1,Pank2 neuronal knockout mice (untreated filled circles; treated, open circles) at day 45 with (open squares) or without (closed squares) PZ-2891 therapy. The p values are in red, and means ± SEM are plotted.

Methods
Materials. d-[1-14C]Pantothenate (specific activity, 55 mCi/mmol) from American Radiolabeled Chemicals; Ni-NTA resin from Qiagen; [3H]ATP from Perkin Elmer (specific activity, 29.8 Ci/mmol); Sypro Orange dye from Thermo Fisher Scientific; pantothenate-free DMEM from Thermo Fisher Scientific; CoA thioesters from Avanti Polar Lipids. All other materials were reagent grade or better.

Dynamic-injection surface plasmon resonance. Experiments were conducted at 20 °C using a SensiQ Pioneer optical biosensor (SensiQ Technologies). His-tagged human PANK3 was immobilized on polycarboxylate hydrogel-coated gold chips preimmobilized with nitriotriacetic acid (HisCap chips; SensiQ Technologies). The chip was primed in chelating buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 50 μM EDTA, 0.005% Tween-20) and was preconditioned at 10 μl/min with three 60 s injections of wash buffer (10 mM HEPES, pH 8.3, 150 mM NaCl, 350 mM EDTA, 0.05% Tween-20) and one 60 s injection of chelating buffer before being charged with a 60 s injection of 500 μM NiCl2 in chelating buffer. After priming into
binding buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM TCEP, 2 mM MgCl₂, 1 mM ATP, 0.005% Tween-20, 3% DMSO), PANK3 was injected until ~ 300–4000 RU of protein were captured. The reference flow cell on the chip was charged with Ni²⁺ without adding protein.

PZ-2891 was prepared in binding buffer, and a gradient of 0–50 or 0–100 nM was injected in duplicate for each concentration at a flow rate of 200 μl/min using the OneStep Injection feature, which exploits Taylor dispersion to generate a concentration gradient that provides a full titration of analyte in a single injection. A series of buffer-only (blank) injections was included to account for instrumental noise. PANK3 was released from the chip with 500 mM imidazole and recaptured for each cycle due to the slow dissociation of the compound. The data were processed with double reference, solvent corrected and analyzed using the Scion Image package Qdat (version 2.5.3.5, SensQ Technologies). Kinetic rate constants (kᵣ, kₑ) were determined by globally fitting the data at all concentrations to a 1:1 (Langmuir) binding model. Equilibrium dissociation constants were calculated as the quotient Kᵣ = kₑ/kᵣ.

PANK activity assays. PANK activity assay was performed in the presence of 0–10 μM compound in a reaction mixture that contained 100 mM Tris-HCl, pH 7.5, 7.5 mM MgCl₂, 1 mM ATP, 45 μM d[1-¹³C]Pantothenic acid (specific activity 22.5 μCi/mmol) and 5 nM of human PANK3. PANK3 concentrations were calculated using the extinction coefficient at 280 nm of 39,225 M⁻¹ cm⁻¹. The assay was linear with time and after 10 min at 37 °C the reaction was stopped by the addition of 4 μl of 10% (v/v) acetic acid. The mixture was spotted onto a DE81 disk, washed with three successive changes of 1% acetic acid in 95% ethanol and protein formation determined by scintillation counting of the dried disc. If the IC₅₀ was determined to be in the nM range then the assay was repeated in the presence of 0.1–0.01 μM to more precisely determine the IC₅₀. All the experiments were repeated twice in duplicate and the data were an average ± data range. 

The experiments mimicking the mixture of ligands present in vivo were performed under different conditions. The reaction mix for the determination of the activity of recombinant PANK3 (100 nM, 100 μM MgCl₂, pH 7.5), 10 mM MgCl₂, 2.5 mM ATP, 45 μM d[1-¹³C]Pantothenic acid (specific activity 22.5 μCi/mmol) and 5 nM of human PANK3. PANK3 concentrations were calculated using the extinction coefficient at 280 nm of 39,225 M⁻¹ cm⁻¹. The assay was linear with time and after 10 min at 37 °C the reaction was stopped by the addition of 4 μl of 10% (v/v) acetic acid. The mixture was spotted onto a DE81 disk, washed with three successive changes of 1% acetic acid in 95% ethanol and protein formation determined by scintillation counting of the dried disc. If the IC₅₀ was determined to be in the nM range then the assay was repeated in the presence of 0.1–0.01 μM to more precisely determine the IC₅₀. All the experiments were repeated twice in duplicate and the data were an average ± data range. 

Human PANK isofrom distribution. The levels of human PANK mRNA were determined by real time qPCR in C3A cells that were grown to a density of 3 × 10⁵ cells/dish and were treated either with 10 μM PZ-2891 or DMSO for 24 h. RNA was isolated immediately using Trizol reagent according to the manufacturer’s instructions (Invitrogen). The cDNA was synthesized using Super Script™ II, random primers and the RNA templates after the removal of genomic DNA by RNase digestion (Invitrogen). The cDNA was amplified recombinant mouse and human PANK1, 2 α-PANK antibody was validated by immunoblotting serial dilutions of the purified protein, protein synthesis, DNA synthesis or lipid synthesis, respectively. The labeling medium contained medium 0.1% dimethylsulfoxide (DMSO); control) or 10 μM of the compound in 0.1% DMSO. Cells were incubated for 24 h, the labeling medium was removed, and cells were washed with phosphate buffered saline and lysed by sonication in 20 mM Tris-HCl, pH 7.5, 2 mM DTT, 5 mM EDTA and 50 mM NaF. The lysates were fractionated by thin-layer chromatography after removal of the cell debris by centrifugation. The samples were spotted on Silica Gel H plates and resolved with 95% ethanol/28% ammonium hydroxide (4/1, v/v). The distribution of radioactivity on the plate was quantified using a Bioscan Imaging (Bioscan, Inc.) system to determine the total cellular radioactivity determined by a Liquid Scintillation Analyzer (PerkinElmer Tri-Carb 9210 TR). Triplicate culture dishes were labeled for each group plus a dish for each group for determination of cell counts and viability using a Nucleocounter (New Brunswick Scientific) according to the manufacturer’s instructions. The experiments were repeated twice in duplicate and the data represented an overall average ± SEM.

Gel filtration chromatography. Three milligrams of each compound in 0.1% DMSO was injected in 20 mM Tris-HCl, pH 7.5, 200 mM NaCl. The gradient was performed under different conditions. The reaction mix for the determination of the activity of recombinant PANK3 (100 nM, 100 μM MgCl₂, pH 7.5), 10 mM MgCl₂, 2.5 mM ATP, 45 μM d[1-¹³C]Pantothenic acid (specific activity 22.5 μCi/mmol) and 5 nM of human PANK3. PANK3 concentrations were calculated using the extinction coefficient at 280 nm of 39,225 M⁻¹ cm⁻¹. The assay was linear with time and after 10 min at 37 °C the reaction was stopped by the addition of 4 μl of 10% (v/v) acetic acid. The mixture was spotted onto a DE81 disk, washed with three successive changes of 1% acetic acid in 95% ethanol and protein formation determined by scintillation counting of the dried disc. If the IC₅₀ was determined to be in the nM range then the assay was repeated in the presence of 0.1–0.01 μM to more precisely determine the IC₅₀. All the experiments were repeated twice in duplicate and the data were an average ± data range.

The temperature was ramped from 25 °C to 95 °C at a rate of 1 °C/min, centrifuged and placed in an ABI 7500 real time PCR system for thermal shift analysis. The temperature was ramped from 25 °C to 95 °C at a rate of 1 °C/min, centrifuged and placed in an ABI 7500 real time PCR system for thermal shift analysis. The temperature was ramped from 25 °C to 95 °C at a rate of 1 °C/min, centrifuged and placed in an ABI 7500 real time PCR system for thermal shift analysis. The temperature was ramped from 25 °C to 95 °C at a rate of 1 °C/min, centrifuged and placed in an ABI 7500 real time PCR system for thermal shift analysis.
PANK1a, PANK1b, PANK2 and PANK3 proteins, and cell lysates expressing each of the isoforms (Supplementary Fig. 17). The entire blots for the cropped data in Fig. 2c,d and Fig. 4 were presented as Supplementary Fig. 18 and Supplementary Fig. 19, respectively.

**Cellular thermal shift.** The method was adapted from Jafari, et al. H2K93T cells were transfected with 5 μg/dish pPANK3, a pcDNA3.1(--)–derived expression vector, in a 100 mm dish. After 24 h the cells were treated with the indicated concentrations of pantarchine or 0.1% DMSO for 2 h at 37 °C. Four dishes were used in each assay. The cells were washed and resuspended in PBS, counted to determine viable cells and then were resuspended in PBS at 5 × 10^6 cells/100 μl aliquots (100 μl) in 0.5 ml reaction tubes were exposed to various temperatures but were not centrifuged to remove the precipitated proteins and probed with anti-β-actin antibody (Sigma-Aldrich). All the experiments were repeated twice in duplicate and the combined data are presented (overall average ± SEM).

**Acetyl-CoA measurement by mass spectrometry.** For acetyl-CoA determinations, C3A cells were resuspended in 1 ml twice in duplicate and the combined data are presented (overall average ± SEM). The blots were blocked for 1 h in 1% milk/TBS-T and then exposed to primary antibody (α-PANK) overnight at 1 μg/ml in 1% BSA/TBS-T followed by secondary antibody (anti-Rabbit AP conjugated) in 1% milk/TBS-T for 1 h at 1:5000 dilution. The blot was washed extensively and exposed to the ECF substrate for 5 min, and the bands on the dried membrane were quantified on the Typhoon FLA9500 using ImageQuant TL software (GE Healthcare). The control blot was done with samples exposed to different temperatures but were not centrifuged to remove the precipitated proteins and probed with anti-β-actin antibody (Sigma-Aldrich). All the experiments were repeated twice in duplicate and the combined data are presented (overall average ± SEM).

**Tissue and cellular CoA determinations.** Cultured cells or frozen tissues were resuspended in 2 ml cold water to which 25 μl 25% KOH was added and derivatized with monobromobimane (mBrB, Life Technologies) and quantified by HPLC23. For acetyl-CoA determinations, C2A cells were resuspended in 1 ml water, added to 2 ml of methanol and 1 ml chloroform, and incubated on ice for 15 min. Chloroform 1.5 ml, 1.2 ml water and 30 ml of [13C6]acetyl-CoA (Sigma) was added, and centrifuged at 2000g for 10 min. The top layer was loaded on a 2- (2-ipyridyl) ethyl solid phase extraction column which was equilibrated with 1 ml 50% methanol/2% acetic acid. The column was washed twice with 1 ml 50% methanol/2% acetic acid and 1 ml water. CoA and thioesters were eluted twice with 1 ml 95% ethanol containing 50 mM ammonium hydroxide. Samples were resuspended in 90% methanol containing 15 mM ammonium hydroxide.

**CoA, dephosphoCoA and phosphophanthetheine determinations.** The mBrB derivatized sample was fractionated by reverse-phase HPLC using a Gemini C18 3 μm column (150 × 4.60 mm) from Phenomenex. The chromatography system was a Waters e2695 separation module with a UV/Vis and fluorescence detector and controlled by Empower 3 software. Solvent A was 50 mM potassium phosphate pH 6.4, and Solvent B was acetonitrile. The flow rate was set at 1 ml/min, the column, and the flow rate was 0.5 ml/min. The HPLC program was the following: starting solvent mixture of 90% A/10% B, 0–2 min isocratic with 10% B, 2–6 min linear gradient from 10% B to 15% B, 6–18 min concave gradient from 15% B to 40% B, 18–23 min isocratic with 40% B, 23–25 min linear gradient from 40% B to 90% B, and 25–28 min isocratic with 90% B. The UV/Vis detector was set at 393 nm, and the fluorescence detector was set with excitation at 393 nm and emission at 470 nm. The elution position of the mBrB-CoA, mBrB-dephospho-CoA (deP-CoA), and mBrB-phosphophanthetheine (PPanSH) were determined by comparison with mBrB-CoA prepared from commercial CoA (Avanti Polar Lipids), mBrB-deP-CoA prepared from commercial deP-CoA (Sigma-Aldrich), and mBrB-PPanSH prepared from a Nudt7-mediated hydrolysis of mBrB-CoA. The areas of the mBrB-derivatized CoA, deP-CoA and PPanSH peaks were integrated and compared to known concentrations of the mBrB-CoA standard.

**Acetyl-CoA measurement by mass spectrometry.** Mass spectrometry of acetyl-CoA was performed using a Finnigan TS Quantum (Thermo Electron) triple-quadrupole mass spectrometer. The instrument was operated in positive mode and the ion source parameters were: ion spray voltage, 5500 V; sheath gas pressure 10, auxiliary gas pressure 5, and tube lens voltage 35 V, sheath gas 50 psi, auxiliary gas 25 psi, and collision energy, 22 V. The system was controlled by the Analyst® software (Sciex) and analyzed with MultiQuant® 3.0.2 software (Sciex).

**Pantothenate extraction and quantification by HPLC/MS/MS.** Plasma (10 μl) was added to 800 μl methanol and 190 μl water along with 200 pmol (β-alanyl-13C4,15N)-pantothenate (13C15N-Pan, Sigma). The mixture (30–40 μg) was homogenized in 2 ml of 80% methanol, and 500 pmol of [13C6]CoA-Pan was added. The samples were incubated at ~80 °C for 4 h. At 3 h, 0.5 mg of [13C6]CoA-Pan was added. Samples were resuspended in water + 0.1% acetic acid and spun through a Spin-X Centrifuge Tube Filter (0.22 μm Cellulose Acetate, Costar). Pantothenate was analyzed using a Shimadzu Prominance UFLC attached to a ScieX QTrap 4500 equipped with a Turbo V ion source. Samples (5 μl) were injected onto an XSelect® HSS C18, 2.1 μl 0.1% acetic acid in water. The ion source parameters were: ion spray voltage, 5500 V; curtain gas, medium; ion source gas 1, 30 psi; and ion source gas 2, 40 psi. The MRM transition for PZ-2891 was 350.2 / 190.0 m/z and warfarin was 309.1 / 163.0 m/z both with a declustering potential, 65 V and collision energy, 30 V. The system was controlled by the Analyst® software (Sciex) and analyzed with MultiQuant® 3.0.2 software (Sciex).

**Degradation of SynCre Pank1, Pank2 neuronal knockout mice.** Generation of the Pank1fl/fl and the Pank2fl/fl mice was reported previously12,13. The SynCre transgene originated in B6.Cg-Tg(Syncre1er7Jm1)J2 transgenic mice (The Jackson Laboratory) that express the Cre recombinase driven by the synapsin 1 promoter; SynCrePank1−/− and SynCrePank2−/− mice were bred with SynCrePank1+/− and SynCrePank2+/− mice for 2 weeks prior to the experiment. The mice were maintained at room temperature 22 °C ± 2 °C, humidity 50 ± 10%, and a light 14/10 h dark cycle with the dark cycle starting at 18:00 h. Water was supplied ad libitum. The mice were randomized into the treatment arms to achieve a normal weight distribution. PZ-2891 was formulated in 30% Captisol®, and was administered by oral gavage at 12 h intervals for 5 doses. The mice were euthanized and tissues harvested 4 h after the last dose. The tissue samples were used for total CoA, pantothenate and pantazine determinations. Blood was collected from euthanized animals, plasma or serum was prepared and stored frozen until analysis. Organs, including liver, forebrain, and hindbrain were quickly excised from euthanized animals and immediately flash frozen in liquid N2 or immersed in RNAlater® (Qiagen) overnight prior to freezing. Forebrain and hindbrain regions were identified40. Total CoA was determined using 20–50 mg of tissue (liver, forebrain or hindbrain) homogenized in 2 ml of 1 mM KOH. The pH was adjusted to 12.0 with 0.25 M KOH, and incubated at 55 °C for 2 h. The pH of the sample was adjusted to 8.0, and the samples were derivatized with monobromobimane (mBrB) and analyzed by HPLC equipped with a fluorescence detector.

**Generation of SynCre Pank1, Pank2 neuronal knockout mice.** Generation of the Pank1fl/fl and the Pank2fl/fl mice was reported previously12,13. The SynCre transgene originated in B6.Cg-Tg(Syncre1er7Jm1)J2 transgenic mice (The Jackson Laboratory) that express the Cre recombinase driven by the synapsin 1 promoter; SynCrePank1−/− and SynCrePank2−/− mice were bred with SynCrePank1+/− and SynCrePank2+/− mice for 2 weeks prior to the experiment. The mice were maintained at room temperature 22 °C ± 2 °C, humidity 50 ± 10%, and a light 14/10 h dark cycle with the dark cycle starting at 18:00 h. Water was supplied ad libitum. The mice were randomized into the treatment arms to achieve a normal weight distribution. PZ-2891 was formulated in 30% Captisol®, and was administered by oral gavage at 12 h intervals for 5 doses. The mice were euthanized and tissues harvested 4 h after the last dose. The tissue samples were used for total CoA, pantothenate and pantazine determinations. Blood was collected from euthanized animals, plasma or serum was prepared and stored frozen until analysis. Organs, including liver, forebrain, and hindbrain were quickly excised from euthanized animals and immediately flash frozen in liquid N2 or immersed in RNAlater® (Qiagen) overnight prior to freezing. Forebrain and hindbrain regions were identified40. Total CoA was determined using 20–50 mg of tissue (liver, forebrain or hindbrain) homogenized in 2 ml of 1 mM KOH. The pH was adjusted to 12.0 with 0.25 M KOH, and incubated at 55 °C for 2 h. The pH of the sample was adjusted to 8.0, and the samples were derivatized with monobromobimane (mBrB) and analyzed by HPLC equipped with a fluorescence detector.
deleted in neuronal tissues. Control littermate mice had the Pank1<sup>fl/fl</sup>,Pank2<sup>fl/fl</sup> SynCre<sup>−</sup> genotype. PCR genotyping primer pairs and products are listed in Supplementary Table 4. PCR analysis was used to genotype tail biopsies where a 338 bp product indicated the floxed Pank1 allele, a 332 bp product indicated the floxed Pank2 allele, and a 285 bp product indicated the presence of the Cre transgene. RNA was isolated from cryo-preserved liver or brain tissue. Synthesis of first-strand cDNA was obtained by reverse transcription using SuperScript<sup>™</sup> III RNase H reverse transcriptase, the RNA templates and random primers. Quantitative real-time PCR was performed in triplicate using the ABI Prism<sup>™</sup>7700 Sequence Detection System with the primers listed in Supplementary Table 5. The Taqman human GAPDH (Applied Biosystems) were used as controls. All of the values were compared using the Ct method<sup>51</sup>, and the amount of cDNA (2−ΔΔCt) was calculated relative to human glyceraldehyde-3-phosphate dehydrogenase mRNA. Nucleotide-specific deletion of the Pank1- and Pank2-floxed alleles was confirmed in brains by the presence of a 218 bp and 176 bp product, respectively, that were absent in liver (Supplementary Figure 14). Incomplete deletion of the floxed alleles in brain was indicated by coincidence of the Pank1-floxed and Pank1-deleted PCR products, and the Pank2-floxed and Pank2-deleted PCR products. Cell types other than neurons contributed to the undeleted, residual Pank1- and Pank2-floxed genes in brain. Wild-type matched control animals were derived from breeding pairs that were heterozygous for both Pank1-floxed and Pank2-floxed alleles and lacked the SynCre transgene. Following genotyping, SynCre<sup>+</sup> and SynCre<sup>−</sup> Pank1-floxed and Pank2-floxed mice regardless of sex were randomly enrolled into the treatment or control arms of the PZ-2891 trial as they emerged from the breeding program.

Open field locomotion. Individual mice were placed in an open rectangular arena (36.8 cm × 43.2 cm) for 5 min during the light phase and motor activity was evaluated using a video tracking system provided by HVS Image with associated software. Each arena was 36.8 cm × 43.2 cm for 5 min during the light phase and the total distance traveled and the percentage of time in motion were recorded. The spontaneous activity of each mouse was measured in the arena under standard overhead lighting and the total distance traveled and the percentage of time spent in the center of the arena was calculated relative to the background.

Crystallization and structure determination. PANK3 with two amino acids (DD) added to the carboxy-termius was expressed, purified and crystallized<sup>60</sup>. The crystals of the PANK3<sup>AMPPNP</sup>Mg<sup>2+</sup>-pantothenate complex<sup>61</sup> were soaked in mother liquor (0.2 mM ammonium acetate, 0.1 mM citrate, pH 5.6, 50 mM MgCl<sub>2</sub>, 32% polyethylene glycol (4 K), 4% DMSO, 10 mM AMPPNP (adenosine 5′-β,γ-methylene)triphosphate) and 1 mM PZ-2891 for two days. The crystals were cryoprotected with 29% ethylene glycol. Diffraction data were collected at the SER-CAT beam line 22-ID at the Advanced Photon Source, and processed with HKL-2000<sup>52</sup>. The structure was solved by molecular replacement using the PANK3 structure (PDB ID: 3SMS) and the program PHASER<sup>53</sup>. The structure was reprocessed using HKL2000<sup>52</sup>. The structure was solved by molecular replacement using the PZ-2891 complex. All structures were rendered with PyMOL<sup>54</sup>. The structure factors have been deposited in the Protein Data Bank as PDB entry 1.72/0.0 for the PZ-2891 complex. All structures were rendered with PyMOL<sup>54</sup>.

Data availability

The PANK3<sup>AMPPNP</sup>Mg<sup>2+</sup>-PZ-2891 crystal structure and diffraction data have been deposited with the worldwide protein data bank under accession code 6B3V [https://doi.org/10.2210/pdb6b3v/pdb].
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Author contributions
All authors contributed to formulating the conclusions, writing the manuscript and approved the text, tables and figures. L.K.S. and R.E.L. designed and interpreted the chemical biology experiments. L.K.S. synthesized the compounds and prepared the formulation for animal studies. S.W.W. and M.-K.Y. designed and interpreted the structural biology experiments. M.W.F. performed the CoA measurements and mass spectrometry. C.O.R. and C.S. designed and interpreted the biochemistry experiments. S.J. derived the animal model and S.J. and C.S. designed and interpreted the cell and animal experiments.

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