Mass Spectrometric Detection and Characterization of Atypical Membrane-Bound Zinc-Sensitive Phosphatases Modulating GABA<sub>A</sub> Receptors

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Abstract

**Background:** GABA<sub>A</sub> receptor (GABA<sub>A</sub>R) function is maintained by an endogenous phosphorylation mechanism for which the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the kinase. This phosphorylation is specific to the long intracellular loop I<sub>2</sub> of the α<sub>1</sub> subunit at two identified serine and threonine residues. The phosphorylation state is opposed by an unknown membrane-bound phosphatase, which inhibition favors the phosphorylated state of the receptor and contributes to the maintenance of its function. In cortical nervous tissue from epileptogenic areas in patients with drug-resistant epilepsies, both the endogenous phosphorylation and the functional state of the GABA<sub>A</sub>R are deficient.

**Methodology/Principal Findings:** The aim of this study is to characterize the membrane-bound phosphatases counteracting the endogenous phosphorylation of GABA<sub>A</sub>R. We have developed a new analytical tool for *in vitro* detection of the phosphatase activities in cortical washed membranes by liquid chromatography coupled to mass spectrometry. The substrates are two synthetic phosphopeptides, each including one of the identified endogenous phosphorylation sites of the I<sub>2</sub> loop of GABA<sub>A</sub>R α<sub>1</sub> subunit. We have shown the presence of multiple and atypical phosphatases sensitive to zinc ions. Patch-clamp studies of the rundown of the GABA<sub>A</sub>R currents on acutely isolated rat pyramidal cells using the phosphatase inhibitor okadaic acid revealed a clear heterogeneity of the phosphatases counteracting the function of the GABA<sub>A</sub>R.

**Conclusion/Significance:** Our results provide new insights on the regulation of GABA<sub>A</sub>R endogenous phosphorylation and function by several and atypical membrane-bound phosphatases specific to the α<sub>1</sub> subunit of the receptor. By identifying specific inhibitors of these enzymes, novel development of antiepileptic drugs in patients with drug-resistant epilepsies may be proposed.

Introduction

Neuronal inhibition is essentially mediated by GABA type A receptors (GABA<sub>A</sub>R) forming anionic channels [1]. They are pentameric oligomers assembled with several subunit classes that may have multiple isoforms [1–3]. In adult rat brain, the most abundant subunits are α<sub>1</sub>, β<sub>2</sub>, and γ<sub>2</sub> typical for 60% to 90% of GABA<sub>A</sub>R [4]. The α<sub>1</sub> subunit is highly expressed throughout most brain regions especially in the cortex [5]. The function of these receptors can be modulated by reversible post-translational modifications such as phosphorylation-dephosphorylation [6–8]. Each subunit has four transmembrane domains and a large intracellular loop (I<sub>2</sub>) between transmembrane domains 3 and 4 [2,9], containing consensus phosphorylation sites for both Ser/Thr and Tyr protein kinases [10–12]. The related modifications by several kinases have multiple effects such as direct modulation of the channel function [13], or receptor trafficking between synaptic sites and intracellular compartments [14,15]. In addition dephosphorylations by protein phosphatases (PP) have been shown to reverse the action of these kinases. For instance, PP1 and PP2B phosphatases dephosphorylate the β1–3 and γ2 subunits [16,17], whereas PP2A dephosphorylates β3 subunits [18,19].

Recently, a new concept of GABAergic inhibition modulation by glycolysis has been described. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) a key glycolytic enzyme has been identified as a kinase for the GABA<sub>A</sub>R α<sub>1</sub> subunits [20]. This endogenous kinase is directly tied to the receptor at the neuronal membrane and phosphorylates the α<sub>1</sub> subunits at two identified serine and threonine residues. This phosphorylation of the α<sub>1</sub> GABA<sub>A</sub>R subunit has been termed “endogenous” since it does not require any exogenous kinase or kinase activator [21].

We have previously shown that this phosphorylation prevents rundown of the GABAergic responses on acutely dissociated pyramidal neurons from rat cortex. In addition, an unknown
related phosphorylation site is present in several GABAAR α subunits and opposes the endogenous phosphorylation [22]. Protein phosphatases modulate the responsiveness of individual synapses to neural activity [16]. Ser/Thr protein phosphatases are expressed in many cell types and cellular compartments, and are regulated via several mechanisms [23]. They are classified into phosphoprotein phosphatases (PPP's) and metal-dependent protein phosphatases (PMP's), families defined by distinct amino acid sequences and 3-D structures.

Alterations in GABAAR expression and function lead to various neurological diseases including epilepsy, anxiety and schizophrenia [24]. Studies carried out on human brain tissue of patients with drug-resistant epilepsies show that both endogenous phosphorylation and function of GABAAR are deficient in the cortical epileptogenic zones [25]. Enhancing the GABAAR endogenous phosphorylation state by inhibiting unknown membrane-bound phosphatase(s) would maintain GABAAR receptor function and therefore prevent seizures to occur. We propose this modulatory mechanism as a new target for the development of antiepileptic molecules active in drug-resistant epilepsies.

We have developed an electrophoresis autoradiographic method to measure the GABAAR endogenous phosphorylation/ dephosphorylation in washed cortical membranes [22]. However, this technique is not suitable to characterize the unidentified membrane phosphatase. The first goal of the present study was to elaborate an alternative approach to detect and characterize the membrane-bound phosphatases. Here, we describe a rapid and highly sensitive method for the quantification of phosphatase activity in washed brain cortical membranes. Further, we aimed at characterizing the kinetics and the pharmacological profile of these enzymes using the novel methodological tool proposed here. We show that the pharmacological profiles of these phosphatases are atypical and do not correspond to classical phosphatases. Our biochemical and functional results bring new insights on these membrane-bound phosphatases specific to GABAAR α1 subunit.

Materials and Methods

Synthetic peptides design

Four peptides were synthesized by Sigma-Genosys the amino acid sequences of which include the two identified endogenous phosphorylation sites of the I2 loop of GABAAR α1 subunit (sequences are identical in the human, rat, bovine and mouse species). The minimal consensus [NXX(T/S)K] of GAPDH-sequences are identical in the human, rat, bovine and mouse PEOCS ONE | www.plosone.org 2 June 2014 | Volume 9 | Issue 6 | e100612

membrane-bound phosphatase dephosphorylates the GABAAR α1 subunit and opposes the endogenous phosphorylation [22]. Protein phosphatases modulate the responsiveness of individual synapses to neural activity [16]. Ser/Thr protein phosphatases are expressed in many cell types and cellular compartments, and are regulated via several mechanisms [23]. They are classified into phosphoprotein phosphatases (PPP's) and metal-dependent protein phosphatases (PMP's), families defined by distinct amino acid sequences and 3-D structures.

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Washed cortical membrane preparation

Washed membranes were prepared mainly from bovine and rat brain cortex. The human cortex sample was frozen on dry ice in the operating room and then thawed for assay. The patient was a girl aged 16 years at the time of curative surgery. She was suffering from epilepsy associated to a non-malignant gangliogioma. The tissue sample was taken outside the tumor in the posterior part of the right inferior temporal gyrus, with information and consent of the patient and of her parents.

The gray matter was homogenized in a Polytron system in 10 volumes of an ice-cold buffer containing 50 mM Tris pH 7.4, 0.32 M sucrose, 5 mM EDTA and 1 mM EGTA. The homogenate was centrifuged at 800–1000 g for 10 min at 4°C. The supernatant (S1) was collected and centrifuged at 100 000 g for 30 min at 4°C. The pellet (P2) was osmotically shocked in 10–20 volumes of ice-cold water and sonicated. The microsome suspension was centrifugated at 100 000 g for 30 min at 4°C. The pellet (P3) corresponding to washed membranes was suspended in a buffer containing 10 mM Hepes (pH 7.4) and 10 mM KCl, and then centrifuged again. The last pellet (P4) was stored at −80°C.

Total protein concentrations were determined using the Bradford-based protein assay reagent (Bio-Rad) with Coomassie Brilliant Blue G-250. Calibration was done with a 20–500 µg/ml BSA range. Absorbance was measured at 570 nm.

Liquid chromatography-electrospray ionization mass spectrometry

Instrumentations. The HPLC-mass spectrometric analysis was performed using a LCQ Advantage ion-trap mass spectrometer (ThermoFinnigan) equipped with electrospray ionization (ESI) source in the positive mode. The ESI source was coupled online with liquid chromatography (nano- or micro-LC) systems.

Nano-HPLC chromatography. Eluted peptides were separated with Ultimate 3000 system (Dionex). The peptide preparations were loaded under different conditions through an autosampler (Dionex) onto a reverse-phase C18 capillary column (packed C18 PepMap 15 cm L×180 µm ID×3 µm dp, 100 Å, Dionex). Separation was achieved with a gradient elution program. The sample injection-loop was of 2 µl when the microcapillary nano-LC system was used. The peptide mixture (native and phosphorylated peptides) was eluted from the column with gradients in the mobile phase from A (5% acetonitrile/0.1% formic acid/94.9% water, v/v/v) to B (80% acetonitrile/0.1% formic acid/19.9% water, v/v/v). LC separation was resolved at the flow rate of 4 µl/min, by the following gradient conditions: 0–4 min 0–20% B, 4–14 min 20–30% B, 14–15 min 30–100% B, 15–17 min 100% B, 17–20 100–0% B. At the end of the run, the column was equilibrated in solvent A for 3 min. The elution solvents were filtered through a membrane filter (type HA 0.45 µm, Millipore).

Micro-HPLC chromatography. Chromatographic separations in micro-flow were conducted on a reversed phase C18 capillary column (150 mm L×2.1 mm ID×5 µm dp, 100 Å, Atlantis, Waters) at a flow of 250 µl/min. Injection loop was of 10 µl. Solvent and gradient are the same as in nano-LC. The chromatographic system is Surveyor MS Pump (ThermoFinnigan) coupled to the autosampler injector Surveyor (ThermoFinnigan).

After separation the eluted peptides were directly electrosprayed into the LCQ mass spectrometer at a voltage of 5 kV. The capillary voltage was 3 V, and the temperature was kept at 280°C. The sheath (helium) and auxiliary gas (nitrogen) flow-rate was set at 35 and 15 (arbitrary units) respectively. Helium was used as a collision gas for fragmentation of ions. A full-scan mass spectrum
indicate the synthetic N- and C-terminal peptides (AA334–346 and AA407–420 respectively) used in this study, with the two identified phosphorylation sites (PO4) on threonine (337Thr) and serine (416Ser) residues involved in the endogenous phosphorylation. EXT, extracellular; INT, intracellular. These peptides are detected in positive mode electrospray ionization (ESI) from m/z 200 to 2000 (mass in Da to charge ratio). The most abundant peptides (in blue) are selected for the subsequent ESI-MS/MS studies (parent peptides) and the other labeled ions have two, three or four charges. MS of native (A), and phosphorylated (B) N-terminal peptides. MS of native (C) and phosphorylated (D) C-terminal peptides. The major fragment ions produced by peptide collision induced dissociation of parent peptide ions are labeled and the nature of fragmentation is indicated by “b” (N-flanking) or “y” (C-flanking) when the ion is broken at peptide bounds (break position in the amino acid sequence) or by losses (+) or gains (+) of small neutral molecules (water, ammonia). The highest intensity peaks used for identification and quantification of the different peptides are indicated in blue. LC-MS/MS analysis was performed simultaneously for the native and phosphorylated peptides. (E) Spectrum of native (F) phosphorylated N-terminal peptides. (G) Spectrum of native (H) phosphorylated C-terminal peptides. The inserts show the chromatograms of the chosen fragmentation products.

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Optimization of LC-MS and LC-MS/MS conditions. The LC-MS conditions were optimized by monitoring chemical parameters of standard N- and C-terminal peptides dissolved in the medium used afterwards for phosphatase activity assays. The buffer medium contained Hepes only (10 to 50 mM; pH 7.3) since Tris decreased signal sensitivity. The Mg2+ ions up to 100 mM did not produce any significant signal decrease. The enzymatic reaction was stopped with ice-cold 10% acetic acid instead of 0.1 M HCl, removing the signals. These conditions enabled us to obtain adequate media for assaying phosphatase activities through measurements of the two native and phosphorylated peptide forms. For the LC-MS/MS method, the collision gas (He) pressure and collision gas voltage were adjusted to get the highest signal of the ionized product for each peptide.

Data analysis. Chromatographic data acquisition, peak integration and quantification were performed using the Xcalibur LC-Quan software package. Mass analysis to determine the nature of fragments in MS/MS was done on the online EXASPy proteomic server using the ‘ProteinProspector’ tool for MS products (http://www.expasy.ch).

Phosphatase assays using phosphopeptides

Phosphatasic activity of bovine, rat and human washed cortical membranes was measured by using synthetic phosphopeptides as substrates. Reactions were performed in presence of 50 μg/ml (total proteins) of washed cortical membrane, 10 mM Hapes-Tris pH 7.4 and 1 mM MgCl2. Enzymatic reactions were initiated by adding 10 μM of phosphopeptide incubated for 10 min at 30°C and stopped by adding ice-cold acetic acid at a final concentration of 10% (v/v). Following a 900 s centrifugation during 10 min at 4°C, the supernatants were analyzed by LC-MS/MS. This centrifugation step was critical to remove membrane suspension that interferes with nano-LC and MS analysis. For these reasons, and to avoid micro-capillary column obstruction we have preferentially used the micro-LC column to characterize the pharmacological profile of phosphatasic activities.

Quantification of appearance of the native peptide and disappearance of the phosphopeptide in different conditions was performed using the calibration curve plotted using the Xcalibur software (P/N 64018-XCALI version 1.5). The calibration curve and linear regression were constructed using a series of standards, native and phosphorylated equimolar mixture, N- or C-terminal peptides at different concentrations 0.5, 1, 2, 5 and 10 μM (n = 2) in 10 mM Hepes pH 7.4, 1 mM Mg2+ and 10% acetic acid, which was the same milieu condition as in the phosphatase assay after stopping reactions. The linear regression represents peak area of MS/MS chromatogram spectra depending on the peptide concentration (Figure S1).

To analyze the impact of washed cortical membranes and incubation process on the native and phosphorylated peptides, calibration standards at different concentrations were prepared in the biological matrix using washed cortical membranes (50 μg/ml of total proteins) in 10 mM Hepes, 1 mM Mg2+ and acetic acid 10% (v/v) incubated at 30°C for 10 min. For phosphopeptides, acetic acid was added before washed cortical membranes in order to prevent the dephosphorylation mechanism (Figure S2). The total amount of the [native + phosphorylated] peptides retrieved after reaction incubations and LC-MS/MS analyses was constant and identical to the phosphopeptide amount added at the starting time (Figure S3).

Enzyme kinetics

The best conditions of incubation time and total protein concentration of washed cortical membranes were determined for assaying phosphatase activity with 10 μM of pL2z1 phosphopeptide. Protein concentration varied from 1 to 300 μg/ml and incubation time from 0.25 to 30 min, at fixed temperature of 30°C as in previous studies [22]. Initial velocity of the enzymatic reaction was determined by linear regression (slope value). The optimal conditions were obtained with 50 μg/ml total membrane protein concentration and 10 min incubation time. The effects of the substrate effects were measured by varying the pL2z1-N-P and pL2z1C-P concentrations between 3 and 75 μM. Saturation plots were generated via non-linear fit to the Michaelis-Menten equation. The affinity constant (Km) and maximum velocity (Vmax) were evaluated from this curve fitting using the Prism 5 computer program (GraphPad). Double-reciprocal plots with the Lineeweaver-Burk equation were also drawn by linear fits using the same software.

Pharmacological profiling of phosphatase activities

Different effectors were tested by addition to the incubation medium at variable concentration: the dicationic ions Mg2+ and Ca2+, Zn2+ were used as chloride, the polycation spermine as hydrochloride, the anion F– as sodium fluoride (NaF), and the inhibitors of some known phosphatases okadaic acid and the two (+/-) enantiomers of β-bromotetramisole oxalate (β-Br-t). For the inhibitory effectors, relative 50% inhibition concentration (IC50) values were either determined graphically with a log-scale for the concentration axis or by the 'log (inhibitor) vs. response' nonlinear fit of Prism 5 software (GraphPad), considering activity in the absence of inhibitor as 100%. However, when Zn2+ was tested with the N and C-terminal peptides as substrates, a step was...
observed that was considered as an intermediate reference value to determine two sequential IC₅₀.

Electrophysiological approach
Adult Sprague Dawley rats (35–40 days of age) were anesthetized by isoflurane and killed by decapitation. The brains were removed quickly and placed in cold artificial cerebro-spinal fluid (Ringer) containing (in mM) 126 NaCl, 3 KCl, 26 NaHCO₃, 1.25 Na₂HPO₄, 2.5 CaCl₂, 1.5 MgCl₂, 10 D-glucose, saturated with carbogen gas (O₂:CO₂, 95:5%) pH 7.4. Cortical neurons were acutely dissociated from 400 μm thick slices by incubation in 3 mg/ml protease-XXIII (Sigma) at 32°C, followed by mechanical dissociation. After washing, the cells were transferred in a solution containing (in mM) 135 NaCl, 3 KCl, 2 CaCl₂, 10 Hpes, 1 MgCl₂, 7 TEA-Cl, 10 D-glucose, and 1 μM TTX, pH 7.4. Pyramidal neurons were recorded using borosilicate glass pipettes (4–5 MΩ) filled with a solution containing (in mM) 130 CsF, 10 CaCl₂, 0.5 CaCl₂, 10 Hpes, 5 EGTA, and 7 Mg-ATP. Okadaic acid at 10 μM was added to this intracellular milieu for specific experiments.

Whole-cell peak currents induced by GABA (100 μM; fast applications of 1 sec pulses every 3 min) were measured for each application and normalized to the maximal response. This maximal response was observed within 0–6 min following the seal of the patch since there was an initial run-up in some cases. The holding potential was −80 mV such that GABA evoked inward currents (Cl⁻ equilibrium potentials were measured close to −40 mV).

Chemicals reagents
Zinc chloride, (−)-p-bromotetramisole oxalate and sodium fluoride were purchased from Fluka Chemika. MgCl₂ was obtained from Prolabo. HPLC grade water phlo and acetonitrile were purchased from Carlo-ERBA Reagents. Formic Acid was purchased from ACS-For analysis. Okadaic acid, (−)-p-bromotetramisole oxalate, spermine, albumin from bovine serum (BSA), plus purchased from ACS-For analysis. Okadaic acid, (−)-p-bromotetramisole oxalate and sodium fluoride were purchased from Fluka Chemika. MgCl₂ was obtained from Prolabo. HPLC grade water phlo and acetonitrile were purchased from Carlo-ERBA Reagents. Formic Acid was purchased from ACS-For analysis. Okadaic acid, (−)-p-bromotetramisole oxalate, spermine, albumin from bovine serum (BSA), plus purchased from ACS-For analysis. Okadaic acid, (−)-p-bromotetramisole oxalate and sodium fluoride were purchased from Fluka Chemika. MgCl₂ was obtained from Prolabo. HPLC grade water phlo and acetonitrile were purchased from Carlo-ERBA Reagents. Formic Acid was purchased from ACS-For analysis. Okadaic acid, (−)-p-bromotetramisole oxalate, spermine, albumin from bovine serum (BSA), plus purchased from ACS-For analysis.

Statistical analysis
Data points are expressed as mean ± standard error to the mean (S.E.M.) of repeated measures (n). Non-linear regressions for the kinetics parameters (and corresponding errors), and statistical comparison between two linear regressions by the ‘slope test’ (case of N-terminal substrate) were assessed using the Prism 6 software (GraphPad). For cluster analysis, ANOVA and post hoc tests, the JMP 10 software (SAS Institute Inc) was used. In all cases the alpha error was set at 5%.

Ethics
All animal experimental procedures were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the Animal Experimentation Committee of Paris Descartes University.

The sample of human brain cortex was taken from a registered collection called “NeurochirÉpilepsie” (DC-2011-1378) affiliated with the biobank CRB-NSPN of Sainte Anne’s Hospital Center (CHSA), with approval of the local ethical committee (CPP Ile-de-France II, September 12th 2011).

Results
The first characterization of the GABAₐR α₁ subunits dephosphorylation were performed using ³²P-autoradiographic measurements on gel electrophoresis [22], a reliable method but too laborious for routine assays. An efficient method was therefore lacking for rapid assays of the membrane-bound phosphatase activity counteracting the endogenous phosphorylation by GADPH. We therefore developed a novel tool.

Analytical tool for phosphatase assays specific to GABAₐR α₁ subunit using phosphopeptides and LC-MS/MS
Designing synthetic peptides for the α₁ subunit of GABAₐR. Two fragments of the mammalian GABAₐR intracellular loop I₂ of the α₁ subunit (I₂α₁; 87 AA-length) including the two phosphorylated sites at threonine and serine residues were synthesized to study the corresponding phosphatases. The N-terminal peptide corresponds to the initial sequence of the I₂α₁ intracellular loop precisely adjacent to the TM3 domain, whereas the C-terminal peptide corresponds to the end-sequence of the I₂α₁ flanking precisely the TM4 domain. Figure 1A insert. For each fragment, a native and a phosphorylated form were synthesized by Sigma-Genosys, with distinct molecular masses. We used these peptides as useful probes to investigate experimental conditions for the retention and separation of the native and phosphorylated forms by reversed-phase liquid chromatography (LC) and electrospray ionization (ESI) for a full scan mass spectrum.

Full-MS detection and characterization of peptides. Sequential mass spectra for simultaneous analyses of N- and C-terminal peptides were obtained from LC-MS using a positive mode full mass scan (full-MS; m/z 200–2000). The native and phosphorylated N-terminal peptides showed that the main ions in the MS spectrum were multiple-protonated molecular ions (M+nHⁿ⁺).

Native (pI₂α₁N, Mr = 1606 Da) and phosphorylated (pI₂α₁N-P, Mr = 1687 Da) N-terminal peptides show respectively dominant triple-charged ions [M+3H]⁺ at m/z 536.0, and 562.8 m/z (Figure 1A, B). Full-MS of C-terminal peptides pI₂α₁C and pI₂α₁C-P with the molecular mass Mr = 1648 Da and Mr = 1730 Da respectively, gave rise to dominant ions at m/z 413.3, a quadruple-charged molecular ions [M+4H]⁺ (Figure 1C) and at m/z 577.1, a triple-charged molecular ions [M+3H]⁺ (Figure 1D). We chose these ions for detection of the peptides in tandem MS (MS/MS).

LC-MS/MS analysis of N- and C-terminal peptides. The parent ions were selected and fragmented for precise peptide identifications. The MS/MS spectrum of N-terminal native and phosphorylated peptides dominant double-charged ions were detected at respectively m/z 702.1 and 741.8, at LC retention times of respectively 8.82 min and 9.67 min (Figure 1E, F and inserts). These ions corresponded to the b₁₁ fragment ions (b₁₁)²⁺ for the native and phosphorylated peptides. The MS/MS products for the native C-terminal peptide showed at m/z 408.7 a dominant fragment ion [M-H₂O+4H]⁺ with four charges and with a loss of one H₂O molecule, while the fragmentation of the phosphopeptide presented at m/z 571.2 a dominant triple-charged ion [M-H₂O+3H]⁺ with a loss of one H₂O molecule (Figure 1G, H). These peptides were eluted at 8.13 min for the native peptide and at 8.37 min for the phosphopeptide (Figure 1G, H inserts). These methods allowed rapid, sensitive and specific simultaneous determinations of the native and phosphorylated forms, qualitatively and quantitatively.

Zn-Sensitive Phosphatases and GABA(A) Receptor
Phosphatase activity assays using the LC-MS/MS method and the phosphopeptides as substrates

We used initially as substrate for phosphatase assays the N-terminal phosphopeptide PI2-N incubated with washed cortical membranes of bovine brain cortex as the enzyme source (Figure 2A, B). In one pilot test we used human brain cortex obtained during neurosurgery to assess that this method can be applied to human frozen tissue (Figure 2C). A specific phosphatase activity was detected in all assays. Figure 2A, B, C shows the disappearance of phosphopeptide (PI2-N-P) versus enzymatic production of native peptide (PI2-N) by varying concentration of total membrane proteins and incubation time. It shows the presence of an important membrane-bound phosphatase activity. The corresponding enzymes(s) recognized and dephosphorylated efficiently the z-subunit substrates, and dephosphorylated the phosphopeptide. The enzyme activity was linear up to 50 μg/ml (total proteins) as shown in Figure 2A. The time course curves showed essentially a linear response up to 10 min (Figure 2B). These parameters (concentration of enzyme and time incubation) were further applied to all the enzymatic assays and related pharmacological studies, using both N- and C-terminal phosphopeptides as substrates.

Enzyme kinetics of membrane-bound phosphatase activities

The phosphatase kinetics parameters were determined with either PI2-N-P or PI2-C-P as synthetic substrates (Figure 2D, E). The results were obtained by fitting data into the Michaelis-Menten equation. The phosphatase activities showed an eightfold reduction in catalytic efficiency (Vmax/Km) for the phosphoserine peptide (PI2-C-P) compared the phosphothreonine peptide (PI2-N-P), due to a combined K_m increase and V_max decrease (Table 1).

The phosphatase activities have two apparent affinities for the N-terminal phosphopeptide as indicated by the Lineweaver-Burk double-reciprocal plot (Figure 2D, insert). The two regression lines of the plot were significantly different as assessed by the slope test (p<0.01 **). This apparent heterogeneity suggests the presence of at least two different species of membrane-bound phosphatases. The phosphatase activities are more efficient at the phosphothreonine peptide. At least one phosphatase species can recognize phosphoserine as a substrate (single apparent slope) (Figure 2E, insert). This feature can be used to discriminate some of the membrane-bound phosphatases.

Pharmacological profiles of membrane-bound phosphatases

The phosphatase activities were inhibited by the ions Zn^{2+}, Ca^{2+} and F^−, Zn^{2+} showing the strongest inhibitory effect. Sodium fluoride, a common inhibitor of PP1, PP2A, and PP2B but not of PP2C or β [26] inhibited phosphatase activities with an IC_{50}<2 mM. Okadaic acid, a potent phosphatase inhibitor of PP1, PP2A and PP2B with distinct IC_{50s} also inhibited membrane-bound phosphatases.

Metal ion dependence of dephosphorylation. We had previously demonstrated that endogenous phosphorylation requires Mg^{2+} ions in the incubation milieu, and that in the presence of these ions GABAAR dephosphorylation occurs spontaneously [22]. Indeed the activities of the PPM family require divalent cations such as Mg^{2+} and Mn^{2+}, Mg^{2+} showing a stronger effect [27]. Other ions such as Ca^{2+} and Mn^{2+} competitively inhibit the phosphatases by blocking the Mg^{2+}/Mn^{2+} binding site [28].

Therefore, assays were performed to determine which divalent cations are effective in modulating the phosphatase activities using the N-terminal phosphopeptide as substrate.

Mg^{2+} ions. Increasing Mg^{2+} concentrations from 1 μM to 1 mM induced a moderate but not linear increase of the phosphatase activities (data not shown). A substantial phosphatase activity was still present without any addition of Mg^{2+}, even when the chelators EDTA (5 mM) and EGTA (1 mM) were added (data not shown), indicating a heterogeneity of the dependence of the membrane-bound phosphatases to this ion. The other cations were tested in presence of 1 mM Mg^{2+}.

Zn^{2+} ions. Previous work [22] showed that Zn^{2+} inhibited membrane-bound phosphatase activity at concentrations between 0.1 μM-1 mM. It was therefore important to confirm this observation by the present method using PI2-N-P and PI2-C-P as substrates (Figure 3). All of the phosphatases activities were completely inhibited by 1 mM Zn^{2+} using either substrate (Figure 3A, 3E). Two apparent IC_{50} for Zn^{2+} were determined for each peptide: high affinity (H) at 1.7 μM (1.4–2.9 μM confidence interval) (Figure 3B) and low affinity (L) at 48 μM (38–60.3 μM) (Figure 3C) for PI2-N-P. For the C-terminal substrate PI2-C-P two affinities were also observed with an IC_{50} (H) = 0.60 μM (0.3–1.3 μM confidence interval) (Figure 3F) for high affinity and an IC_{50} (L) = 15.0 μM (11.5–18.2 μM) for low affinity (Figure 3G). With low concentrations of Zn^{2+} (1 μM-200 nM), we observed several activations of the phosphatase activities using PI2-N-P (Figure 3D), of lower extent for the C-terminal substrate (Figure 3H).

Okadaic acid inhibition. Okadaic acid has been shown to be a potent inhibitor for PP1, PP2A, PP4 and PP5 and a weaker inhibitor for PP2B. PP2C and PP7 are not or very slightly sensitive to inhibition by okadaic acid at micromolar concentrations [29]. The differences in the IC_{50} can distinguish these phosphatases. Okadaic acid at 300 nM completely inhibits the membrane-bound phosphatases using the two phosphopeptides (Figure 4A, B). Two apparent IC_{50} for the inhibition of phosphatase activities by okadaic acid using PI2-N-P were determined: a high affinity (H) at 0.15 nM and a low affinity (L) at 5 nM (Figure 4A). Only one affinity was measured using PI2-C-P with an apparent IC_{50} at 0.18 nM (Figure 4B). These data paralleled those obtained with Zn^{2+} and confirm the presence of at least two species of membrane-bound phosphatases.

Inhibition of the phosphatase activities by (+/-)-p-Br-t oxalate. The levamisole, (−) enantiomer of the β-bromotetramisole oxalate ((−)-p-Br-t) is a potent alkaline phosphatase inhibitor, whereas the same enzyme is completely insensitive to the (+) enantiomer (+)-p-Br-t which is often used as a negative control [30]. We therefore tested both enantiomers with increasing concentrations (Figure 4C). The membrane phosphatase activities were completely inhibited by both enantiomers at 1 mM, with IC_{50} values of 0.1 mM for (−)-p-Br-t and 0.3 mM for (+)-p-Br-t, showing that the alkaline phosphatase is not involved in the dephosphorylation of the N-terminal phosphopeptide.

Inhibition by other ions. By the N-terminal phosphopeptide method, fluoride ([F ] ) reduced phosphatase activities by 45% at 1 mM and almost 90% inhibition was observed at 10 mM, indicating an IC_{50} in the order of 2 mM. Ca^{2+} inhibited phosphatase activities at 1 mM by only 30% and by slightly less than 60% at 10 mM indicating an IC_{50} in the order of 5 mM, which is much less potent than inhibition by Zn^{2+} (Figure 4D).

Polycation inhibition. Some mammalian phosphatases are inhibited by various polycations [26]. The effects of spermine (a tetracationic polyamine) vary from one enzyme to another. Some protein tyrosine phosphatases are stimulated [31–33], and other
Figure 2. LC-MS/MS detection and kinetics analysis of GABAAR α1-subunit phosphatase activities. (A) Dose-dependency at various protein concentrations; (B) time course of phosphatase activities using 50 μg/ml of total proteins of washed cortical membranes from bovine brain and (C) from human epileptic tissue. The N-terminal phosphopeptide (pI2α1N-P) used as substrate at 10 μM was incubated during 10 min at 30 °C in 10 mM Hepes (pH 7.4) in the presence of 1 mM Mg2+. The enzymatic reactions were stopped with 10% acetic acid and the samples were analyzed by LC-MS/MS as in figure 1E, F. The dephosphorylation rate was quantified by measuring simultaneously the quantity of the produced native peptide (pI2α1N) and of the remaining phosphorylated substrate (pI2α1N-P). Error bars represent SEM of two experiments. Kinetic analysis of GABAAR α1-subunit phosphatase activities was investigated using N-terminal (pI2α1N-P) and C-terminal (pI2α1C-P) phosphopeptides. All experiments were carried out with 50 μg/ml membrane proteins concentration, incubated at 30 °C during 10 min, in presence of different concentrations of substrates. (D) Saturation plot of the initial velocity V, versus [pI2α1N-P] and Lineweaver-Burk plots of 1/V versus 1/[pI2α1N-P] (inset) showing two significantly different slopes. (E) Saturation plot of V, versus [pI2α1C-P] and Lineweaver-Burk plot of 1/V versus 1/[pI2α1C-P] (inset). The data points are means ± SEM of 3 experiments.

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Effects of the okadaic acid on the rundown of GABA<sub>A</sub> currents in acutely isolated cortical neurons

We measured the rundown of the GABA-induced currents in pyramidal neurons acutely dissociated from rat cortex (Figure 5A). Rundown of GABA<sub>A</sub> currents is a time-dependent response decrease due to a dephosphorylation process [7] (Figure 5B), which does not require the presence of the receptor agonist GABA, thus differing from the observed desensitization process during GABA applications. We had previously shown that the phosphatase inhibitor okadaic acid (10 μM) added to the pipette milieu has a variable effect on the GABA<sub>A</sub> current rundown in four neurons [20]. As illustrated in Figure 5C, the rundown could be abolished in some cells. To clarify this variable effect, okadaic acid was assayed on a larger number of rat pyramidal cells (n = 17). Normalized maximal responses of neurons displayed different effects with okadaic acid (Figure 5D). We applied the hierarchical clusters test to the parameter of the 'mean normalized currents' (from t = 3 to t = 30 min) for all okadaic acid-treated neurons. Four groups of neurons were significantly distinguished by ranking rundown velocity as ‘Very Rapid’ – ‘Mid Rapid’ – ‘Mid Slow’ – ‘Very Slow’. In addition, the whole group of okadaic acid-treated cells was significantly different from the control cells group (p<0.01 **), using a two-tailed Student’s t-test assuming unequal variances, in spite of the broad individual variation induced by addition of the phosphatase inhibitor. The 4 groups were considered separately using one-way ANOVA with Dunnett’s post hoc test: the ‘Very Rapid’ group which was not sensitive to okadaic acid was identical to control (Ns), and the 3 other groups of neurons which responded to okadaic acid were statistically different from the control (p<0.01 ** for ‘Mid Rapid’, p<0.0001 *** for both ‘Mid Slow’ and ‘Very Slow’). This functional approach thus also shows heterogeneity in terms of phosphatase activities in pyramidal neurons.

Discussion

We have developed an analytical tool to characterize for the first time the membrane-bound GABA<sub>A</sub>-R α1-specific phosphatase activities, which were never previously identified or characterized. These phosphatases can be considered as novel targets for antiepileptic drugs research. Indeed, in recent clinical studies it was proposed to modulate the activity of protein phosphatases for therapeutic benefits [38].

Improved LC-MS-based techniques led to reliable alternatives for peptide analysis and protein quantification [39]. A known limitation in proteomic analysis of membrane proteins by mass spectrometry is the difficulty raised by their amphipathic nature [40]. Thus, detection and characterization of phosphatase activities in membrane preparations by LC-MS/MS analysis and phosphopeptides as substrate is a method not used until now, and its development was a challenge.

We used two fragments specific for the intracellular loop of α1 subunits, which can be used for all mammals. The principle of the present phosphatase assay by LC-MS/MS is based on the measurement of relative MS signals of both phosphopeptides and native peptides resulting from dephosphorylation. We initially characterized these peptides by examining their specific MS/MS spectra of the related dominant ions. The advantage of this method was to analyze simultaneously native and phosphorylated peptides. Assaying washed brain membranes including human cortical membranes as a source of phosphatase activity we observed an important dephosphorylation by transformation of N- and C-terminal phosphopeptides into native peptides, showing thus that the membrane-bound phosphatases recognized our L<sub>γ</sub>1 probes as substrates. This result was crucial since it was a challenge to study such a complex membrane proteins preparation and was a prerequisite to characterize these phosphatases.

A comparative analysis of kinetics parameters of these phosphatases (Table 1) showed that the N-terminal substrate was more efficient (higher specific activity and higher affinity) than the C-terminal one. For the N-terminal phosphopeptide the Lineweaver-Burk plot was not linear. This point was further studied using slope tests: two significantly different apparent affinities were observed. We noted a reduction of the phosphatase heterogeneity of C-terminal comparing to the N-terminal substrate. This was confirmed by the data obtained by okadaic acid inhibition, where we observed two distinct affinities for N-terminal substrate and only one apparent affinity for C-terminal. It has been shown that some phosphatases have indeed a better substrate preference for phosphothreonine than for phosphoserine residues in short peptides [41]. These observations strongly support the hypothesis of several phosphatases regulating the GABA<sub>A</sub>R function.

The protein phosphatases are generally classified according to their substrate specificity, ion requirement and sensitivity to inhibitors, and are divided into two major categories-protein: serine/threonine phosphatases (PSTPs) [42] and tyrosine phosphatases (PTPs) which include dual-specificity phosphatases (DUSPs) [43]. Two distinct PSTP gene families have been described: PPM and PPP. The protein Ser/Thr phosphatases PP1, PP2A, and PP2B of the PPP family, together with PP2C of the PPM family, account for the majority of the protein serine/threonine phosphatase activity. The PPM family is composed of Mg<sup>2+</sup>-dependent phosphatase including PP2C, pyruvate dehydro-

Table 1. Kinetics parameters of the phosphatase activities dephosphorylating the N- and C-terminal phosphopeptides.

| Substrate Name | Peptide sequence | V<sub>max</sub> (pM.min<sup>-1</sup>.ml<sup>-1</sup>) | K<sub>a</sub>(μM) | Efficiency V<sub>max</sub> K<sub>a</sub><sup>-1</sup> (pM.min<sup>-1</sup>.ml<sup>-1</sup>.

Experimental conditions are detailed in Materials and Methods. Values are means ± SEM of two or three experiments.

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Figure 3. Inhibition profiles of the phosphatase (PPase) activities by Zn$^{2+}$ ions using N-and C-terminal phosphopeptides as substrate. Assays were performed in presence of rat washed cortical membrane at 50 μg/ml of total protein concentration, with increasing amounts of Zn$^{2+}$ ions in 10 mM Hepes buffer pH 7.4 with 1 mM Mg$^{2+}$ in presence of. Incubations were performed at 30°C during 10 min. The activities are...
genase phosphatase, and PP2C-“like” phosphatases [44]. These enzymes play a key role in neuronal plasticity, learning and memory [16], and in several neurological disorders such as amyotrophic lateral sclerosis and epilepsy [45]. An important issue is to identify the metal ions required for optimal phosphatase activity. Both PP1 and PP2A are active in the absence of divalent cations, whereas PP2B and PP2C have respectively an absolute requirement for Ca$^{2+}$ and Mg$^{2+}$ [36].

The activity of membrane-bound phosphatases was tested in the presence of a variety of factors that can interfere with these enzymes. Addition of increasing concentrations of Mg$^{2+}$ exhibited moderate effects on activation of phosphatase activities, and in the presence of chelators a residual phosphatase activity was observed, revealing the presence of both Mg$^{2+}$-dependent and Mg$^{2+}$-independent protein phosphatases, indicating again the heterogeneity of these enzymes. A similar heterogeneity was reported for the atrial natriuretic peptide receptor (NPR-A) phosphorylated on 4 serine and 2 threonine residues by an unknown kinase(s) and dephosphorylated by two unknown phosphatases, the activity of which was dependent or not on Mg$^{2+}$ [46].

Ca$^{2+}$, fluoride and spermine at millimolar concentrations inhibited the phosphatase activities. The weak inhibition by spermine was interesting since its content is increased in the epileptogenic tissue [37]. One may speculate that such inhibition could counteract the increased GABA$\_A$ current rundown observed in neurons isolated from human epileptogenic cortical tissue. However, the very high IC$_{50}$ (>10 mM) of spermine inhibition makes such compensation unlikely, since millimolar spermine...
concentrations were never observed in the human cortical tissue, even in pathological conditions. Inhibition observed with \( p \)-bromotetramisole oxalate (\( p \)-Br-t) allowed us to discard the alkaline phosphatase as an involved enzyme in the present mechanism. It has been shown that \( p \)-Br-t can inhibit other phosphatases such as PP1, PP2A, PP2B and PP2C with variable efficacy [30]. The same authors showed an inhibition by both enantiomers for PP2A and PP2C, however with different IC\(_{50}\) in the millimolar range. They also observed that the (+) enantiomer is more potent than the (−) enantiomer for PP2C. Likewise, we showed that inhibition by (+)-\( p \)-Br-t was slightly more effective than that by (−)-\( p \)-Br-t for the membrane-bound phosphatase activity using the N-terminal phosphopeptide. However, the IC\(_{50}\) we measured were 3–7 fold lower than those for PP2C and PP2A. Thus these classical phosphatases were very unlikely involved in the GABAergic \( \alpha 1 \) mechanism.

We had previously showed that sodium orthovanadate inhibits membrane phosphatases [22] and reduced rundown of GABA\(_A\) currents [20]. This phosphate analog could not be used in our conditions for phosphatase characterization, because a complete ESI-MS/MS signal extinction occurred in positive mode occurred, likely due to the negative charge and non-volatility of the VO\(_4^{3−}\) anion. Indeed, all ESI-MS/MS manufacturers totally prohibit the use of phosphate buffers for the same reason [47].

It was of special interest to investigate the zinc-sensitivity of the membrane-bound phosphatase, since zinc oxide was known to present anti-epileptic effects and used for this purpose during the 19th century [48]. The effect of Zn\(^{2+}\) is variable depending on the phosphatases: these ions as well as Fe\(^{2+}\) are activators at the catalytic site of PP1, considered as a metallo-enzyme [49], whereas Zn\(^{2+}\) inhibits the PP2C class by competition with Mg\(^{2+}/\)Mn\(^{2+}\) at the metal binding site of the catalytic domain [26,28]. We had previously shown in washed brain membranes that complete

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**Figure 5. Effects of okadaic acid on the GABA\(_A\) current rundown.** GABA\(_A\) currents were measured by whole-cell patch clamp on acutely dissociated cortical pyramidal neurons from Sprague Dawley rats. (A) Micrographs of a patched cell at two different magnifications, showing the rapid application device (left). (B) Rundown in cortical neuron: GABA was applied at a concentration of 100 \( \mu M \) during 1 second every 3 min. The maximal amplitude of GABAergic currents gradually decreased with time in control conditions. In presence of okadaic acid (10 \( \mu M \) in the pipette) variable effects were observed. (C) In some cells the rundown was even totally abolished. (D Left) Rundown profiles of normalized currents in presence (\( n = 17 \)) or in absence (Control, \( n = 11 \)) of okadaic acid; (D Right) color-coded hierarchical clustering tree for the recorded okadaic acid-treated cells in which a maximum of 4 groups are significantly distinguished: ‘Very Slow’ (\( n = 3 \)), ‘Mid Slow’ (\( n = 5 \)), ‘Mid Rapid’ (\( n = 4 \)) and ‘Very Rapid’ (\( n = 5 \)). For each group the plot (Left) is the average of normalized currents. Error bars indicate the SEM. One-way ANOVA with Dunnett’s test of mean currents and Student \( t \)-test were used (see in Results) indicating very likely that more than one phosphatase are involved. doi:10.1371/journal.pone.0100612.g005
inhibition of GABAₐ, R dephosphorylation occurred with Zn²⁺ using the autoradiographic method [22]. In the present study we found that Zn²⁺ in the micro- to millimolar ranges was one of the most potent tested phosphatase inhibitors. Two apparent affinities were determined using both N- and C-terminal phosphopeptides. Free cellular Zn²⁺ concentrations (picomolar range) are of magnitudes lower than the total cellular Zn²⁺ content (nanomolar range) [50]. In these ranges, we observed a series of activations of phosphatase activities for the N-terminal substrate. Our washed cortical membrane preparations are cytosol free, without proteins chelating Zn²⁺ such as metallothioneins [51]. Therefore, the observed multiple activations of phosphatase activities by zinc are rather reflecting different affinities of distinct phosphatases for these ions. These data indicate again the heterogeneity of the membrane-bound phosphatases dephosphorylating GABAₐ, R 1 subunits. Unfortunately, the effect of Zn²⁺ could not be tested directly on GABAₐ, receptor currents in whole-cell patch-clamp of acutely isolated pyramidal neurons because Zn²⁺ addition into the pipette milieu systematically induced breakdown of the nano-seal at the tip of the glass micropipettes (data not shown).

The organic inhibitors such as okadaic acid are very useful to distinguish some classes of protein phosphatases [32]. Okadaic acid inhibits various phosphatases in mammalian brain with very distinct IC₅₀ values: PP1 (270 nM), PP2A (2 nM), PP2B (3.6 μM), PP4 (0.2 nM), PP5 (1.4–10 nM) and PP6 (2 nM) whereas PP2C, PTP, PP7 acid phosphatase and alkaline phosphatase were not inhibited by up to 1–10 μM okadaic acid [36]. We showed that it strongly inhibited phosphatase activities but the apparent IC₅₀ values were different from those described for the classical phosphatases, suggesting that the membrane-bound phosphatases are not yet identified.

The rundown of the GABAₐ, mediated responses in dissociated neurons involves an endogenous phosphorylation–dephosphorylation process [7,20]. Okadaic acid at 10 μM was tested on the GABAₐ, receptor rundown. Four groups of cortical neurons were distinguished suggesting that each group could correspond to distinct phosphatase types. The group showing no inhibition indicates the presence in these cells of okadaic-resistant phosphatase activity. The 3 groups with different sensitivities to this inhibitor suggest the presence of at least two distinct classes of phosphatases, a further argument for their heterogeneity. In addition, okadaic acid at 10 μM inhibited most phosphatase activities by the phosphopeptide method. This observation suggests that in the course of preparation of the washed cortical membranes, a loss of some GABAₐ, receptor subunit phosphatases weakly attached at the membranes occurred.

We therefore clearly propose heterogeneity of the phosphatases regulating GABAₐ, R endogenous phosphorylation at the cellular level. In addition to the above-mentioned case of NPR-A, other examples of dephosphorylation of a single protein by multiple distinct Ser/Thr phosphatases are known, such as centrin [53] and [54]. The 1-subunit dephosphorylation by multiple phosphatases in GABAₐ, R likely reflects alternate modulations of the inhibitory neurotransmission, and also different functional responses to ions and endogenous effectors in neurons. Since the involved phosphatases do not correspond to the classical enzymes, it will be of great interest to isolate these membrane-bound phosphatases and to identify them in a future study.

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Conclusion and prospects

We have developed an analytical tool using LC-MS/MS method to study membrane-bound phosphatases specific to GABAₐ, R, including those prepared from human brain tissue. This method is highly sensitive and can be applied to crude extracts as well as to purified enzymes and would be useful for membrane phosphatases assays. Our main finding is the heterogeneity of the phosphatases dephosphorylating the GABAₐ, R 1 subunit. Atypical pharmacological profiles were obtained for these phosphatases as shown by the zinc-sensitivity, excluding classical phosphatases even though sharing some of their properties. A challenge for future studies will be to purify these different membrane-bound phosphatases using the present LC-MS/MS method for monitoring. Identifying the membrane-bound phosphatases of GABAₐ, receptors will improve our understanding of the molecular mechanisms modulating GABAergic function and their alterations in patients with drug-resistant seizures. These advances would also provide the basis for development of new antiepileptic drugs by targeting the phosphatases specific to the GABAₐ, R 1 subunit.

Supporting Information

Figure S1 Quantification standard curves of peptides under incubation conditions of phosphatase activity. Native (A) and phosphorylated (B) N-terminal peptides, (C) native and (D) phosphorylated C-terminal peptides were prepared at different concentrations in 10 mM Hepes buffer, 1 mM Mg⁡²⁺ and acetic acid 10% (w/v). Regression lines present chromatogram peak area of MS/MS spectra depending on peptides concentrations. Determination coefficient (r²) was calculated using GraphPad Prism 5. (TIF)

Figure S2 Quantification standard curves of N-terminal peptides in biological matrix using washed cortical membranes. Native (A) and phosphorylated (B) N-terminal peptides were prepared at different concentrations in presence of 50 μg/ml (total proteins) of washed cortical membrane in 10 mM Hepes-Tris buffer pH 7.4 with 1 mM MgCl₂ incubated at 30°C for 10 min, the same conditions as for phosphatase assays. For phosphopeptide, acetic acid 10% (w/v) was added before the membrane preparation in order to prevent dephosphorylation. (TIF)

Figure S3 Double quantification of N-terminal phospho- and native peptide concentrations after dephosphorylation. Phosphatase reactions were performed in presence of N-terminal phosphopeptide (10 μM) at different protein concentrations of washed cortical membranes (1-3-10-30-100 and 300 μg/ml). The total amount (by 2-μl sample) of phosphopeptide and of native peptide was constant, with an average of 20.43 pmol (SEM = 0.32, n = 6). (TIF)

Author Contributions

Conceived and designed the experiments: JJL, RP, JL, PS. Performed the experiments: MS, IK, CR, JJL. Analyzed the data: JJL, RP, MS, CR, IK. Contributed reagents/materials/analysis tools: MS, CR, JJL. Wrote the paper: MS, JJL, RP.
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