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Specific binding of photoaffinity-labeling peptidomimetics of Pro-Leu-Gly-NH_2 to the dopamine D_{2L} receptor: Evidence for the allosteric modulation of the dopamine receptor

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Abstract

The present study was undertaken to investigate the mechanistic role of L-prolyl-L-leucyl-glycinamide (PLG) in modulating agonist binding to the dopamine D_{2L} receptor. Competition and displacement assays indicate that the photoaffinity-labeling peptidomimetics of PLG, 3(R)-[(4(S)-(4-azido-2-hydroxy-benzoyl) amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1a**) and 3(R)-[(4(S)-(4-azido-2-hydroxy-5-iodo-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1b)** bind at the same site as PLG. Autoradiography was used to establish the covalent binding of [^{125}I]-**1b** to a 51 kDa protein in bovine striatal membranes. Western blot analysis with a dopamine D_{2L} specific antibody, in combination with autoradiography, following a two dimensional gel separation, suggested this 51 kDa protein to be the dopamine D_{2L} receptor. Further evidence for binding of **1b** to dopamine D_{2L} was provided by samples immunoprecipitated with the D_{2L} antibody. These samples were analyzed by western blotting in parallel with autoradiography of [^{125}I]-**1b** labeled protein. Both methods revealed bands at 51 kDa. Furthermore, PLG is shown to compete with **1b** for binding to the dopamine D_{2L} receptor as determined by autoradiography, as well as competition experiments with PLG and **1a**. Collectively, these findings suggest the successful development of a photoaffinity labeling agent, compound **1b**, that has been used to elucidate the interaction of PLG specifically with the dopamine D_{2L} receptor.

Keywords

Dopamine D_2 receptor; L-Prolyl-L-leucyl-glycinamide (PLG); melanocyte-stimulating hormone (MSH) inhibiting factor-1 (MIF-1); allosteric; autoradiography; peptidomimetic

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1. INTRODUCTION

The tripeptide L-prolyl-L-leucyl-glycinamide (PLG, Fig 1), also known as melanocyte stimulating hormone release inhibiting factor (MIF-1), is an endogenous brain peptide that has been implicated in modulating dopaminergic neural transmission in the nigrostriatal pathway (Reed et al., 1994;Srivastava et al., 1988). Studies have shown that PLG does not modulate dopaminergic neurotransmission by affecting dopamine synthesis, uptake, or metabolism; rather it functions through a mechanism in which PLG renders the dopamine receptor more responsive to agonists. PLG has been shown to enhance the binding of various agonists such as apomorphine, 2-amino-6,7-dihydroxy-1,2,3,4,-tetrahydronaphthalene (ADTN), and N-propylnorapomorphine (NPA) to striatal dopamine receptors, without altering antagonist binding (Bhargava, 1983;Srivastava et al., 1988). Moreover, a previous study with bovine striatal synaptosomal membranes showed that PLG and its peptidomimetics exhibit their greatest modulatory effect on the dopamine D_{2L} receptor subtype (Verma et al., 2005).

PLG has been shown to possess a variety of pharmacological activities in the central nervous system (Drucker et al., 1994; Hara and Kastin, 1986a; Hara and Kastin, 1986b; Mishra et al., 1983; Reed et al., 1994; Saleh and Kostrzewa, 1989; Srivastava et al., 1988). A series of earlier clinical studies demonstrated that this tripeptide has substantial therapeutic activity in the treatment of Parkinson's disease, antipsychotic drug-induced tardive dyskinesia, and depression (Barbeau, 1978; Ehrensing et al., 1977; Ehrensing et al., 1994). Although PLG is known to attenuate some symptoms of Parkinson's disease (Barbeau, 1975; Kastin and Barbeau, 1972) and tardive dyskinesia (Ehrensing et al., 1977), its short biological half-life has limited its clinical efficacy (Mishra et al., 1997). Therefore, a number of conformationally constrained analogues of PLG have been designed with improved pharmacological properties (Mishra et al., 1997).

Despite the years of study conducted on PLG and its dopaminergic modulatory effects, the mechanistic action of this tripeptide is still unknown. It has been suggested that PLG functions similar to an allosteric modulator (Costain et al., 1999). Support for such a hypothesis was based upon the fact that PLG increases the affinity of the dopamine receptor for its agonist, but it does not behave like an agonist itself (Chiu et al., 1981). However, such activity is also consistent with a mechanism by which PLG interacts with an independent macromolecule that is somehow coupled with the dopamine receptor.

In order to identify the PLG binding site and to shed light on its mechanism of action, we previously designed and synthesized a family of photoaffinity-labeling agents utilizing as a template the potent PLG γ-lactam peptidomimetic, 3(R)-[(2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide (PAOPA, Fig 1) (Fisher et al., 2006). The results from this study provided strong support for the binding of these photoaffinity-labeling agents at the same modulatory site as PLG and its peptidomimetics. In the present study, the photoaffinity labeling agent, 3(R)-[(4(S)-(4-azido-2-hydroxy-5-iodo-benzoyl) amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1b**, Fig 1) has been used in bovine striatal membranes to demonstrate that it is the dopamine D_{2L} receptor with which the PLG peptidomimetic photoaffinity-labeling agent, and thus PLG, interacts.

2. MATERIALS AND METHODS

2.1 Materials

L-Prolyl-L-leucyl-glycinamide (PLG), protein A agarose beads, dopamine and other routine chemicals were purchased from SIGMA Chemical Co. (St. Louis, MO, USA). The PLG

peptidomimetic photoaffinity-labeling ligands 3(R)-[(4(S)-(4-azido-2-hydroxy-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1a,** Fig 1**)** and 3(R)-[(4(S)-(4-azido-2-hydroxy-5-iodo-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1b,** Fig 1) were synthesized as described previously (Fisher et al., 2006). Rabbit anti-dopamine D_{2L} antibody was purchased from Research and Diagnostics (Concord, MA, USA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody was purchased from GE Healthcare Life Sciences (Baie d'Urfe, QC). C18 (18%) chromatographic columns were obtained from Life Science (Peterborough, ON, Canada), IODO-BEADS were purchased from Biolynx Inc. (Peterborough, ON, Canada), and Na-[^{125}I] was obtained from McMaster Nuclear Reactor (Hamilton, ON, Canada). Anti-dopamine D_{2L} receptor antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-goat Alexa Fluor 488 secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA). Anti-rabbit TRITC and the VectaShield mounting media were purchased from Vector Labs (Burlingame, CA, USA). Urea, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), iodoacetamide (IAA), tributylphosphine (TBP), IPG strips, and PROTEAN IEF cell were purchased from Bio-Rad (Mississauga, ON, Canada). Tris (hydroxymethyl)aminomethane, Triton X-100, Coomassie Brilliant Blue G, DL-dithiothreitol (DTT), β-mercaptoethanol, ammonium sulfate, and bromophenol blue were from Sigma (Oakville, ON, Canada).

2.2 Iodination of Photoaffinity Labeling Agent 1a

One C18 (18%) column was equilibrated twice with 5 ml of methanol and twice with 5 ml of distilled water. Two IODO-BEADS were washed twice with 1ml of PBS for 5 min. 1 mg of PLG peptidomimetic photoaffinity labeling agent **1a** was incubated with the IODO-BEADS and 10 μl (1 mCi) of Na-[^{125}I] for 15 min at room temperature with shaking in between. The mixture was then transferred to the pre-equilibrated column and the contents were collected. The column was first washed five times with 1 ml of distilled water, then 1 ml of 50% aqueous methanol followed by 1 mL of 100% methanol. Flow was collected and 2 μl of each collection was counted for radioactivity.

2.3 Competitive Receptor Binding Assay

Competition binding assays were carried out in triplicates using 100 μg of bovine striatal membranes were incubated with 2 nM [^{125}I]-**1b** and increasing concentrations of PLG. The mixture was incubated in a total 1mL volume of assay buffer (50 mM Tris-HCl, 5 mM KCl, 4 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA) and 120 mM NaCl (pH 7.6)), for 8 h at 4°C and terminated by rapid filtration. Radioactivity-bound filter disks were placed in plastic scintillation vials containing 5ml scintillation fluid and were counted in a Beckman LS5000 liquid scintillation counter (model LS5 KTA). Specific binding was defined as the difference between the radioactivity bound in the absence and presence of 1 μM dopamine.

2.4 Photoaffinity labeling with 1b

Membranes were incubated with 1 nM [^{125}I]-**1b** for 1 h at 24°C in the dark, in 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, $MgCl_2$, 0.1 mM PMSF, 1% BSA. After incubation the samples were subjected to irradiation with a UV lamp (350 nm) for 5 min at 4°C. Centrifugation at 16,600 x g for 30 min produced a pellet which was then resuspended in sample buffer (0.625 M Tris, 2% SDS, 10% glycerol, 0.05% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and boiled for 5 min prior to separation on a 12% SDS-PAGE. Alternatively, samples were resuspended in a rehydration buffer (7 M urea, 4% CHAPS, 0.2% 3/10 carrier ampholytes, 0.0002% bromophenol blue and distilled water) for a 2-dimensional gel separation on IPG strips at respective pHs followed by 12% SDS-PAGE separation. The

gels were either stained with Coomassie R-250 and used for autoradiography or transferred to nitrocellulose membrane for western blotting. Autoradiographs were produced by subsequently drying the gel using a Biorad water-vacuum gel dryer (Biorad, Mississauga, ON, Canada) and exposure to Kodak X-OMAT film for 120 h at −80 °C.

2.5 Two Dimensional Gel Separation

Samples suspended in rehydration buffer (8 M Urea, 4% CHAPS, 50 mM DTT, 0.2% carrier ampholytes, 0.0002% Bromophenol Blue) were sonicated and allowed to sit at room temperature for 1 h. Samples were hydrated into respective IPG strips overnight at room temperature. After isoelectric focusing (IEF), the strips were equilibrated at room temperature in equilibration buffer (6 M urea (w/v), 20% SDS (w/v), 1.5 M Tris HCl, pH 8.8, 50% (v/v) glycerol) containing 2% DTT (w/v) and then 2.5% iodoacetamide (w/v). The IPG strips were then subjected to the second dimension SDS-PAGE and run at 100 V for 2 h (Chevallet et al., 1998; Ross et al., 1993). The gels were either stained with coomassie R-250 and used for autoradiography or transferred to nitrocellulose membrane for western blotting.

2.6 Western Blotting

Western blotting was performed as described previously (Chevallet et al., 1998; Ross et al., 1993). After transferring the proteins to nitrocellulose membrane (Hybond ECL 100% Pure Nitrocellulose Membranes), the membranes were blocked with 10% milk buffer (0.05 mM Tris, 0.15 M NaCl, 0.2% Tween-20, pH 8.5 (TBS-T) for 1 h. The blot was then incubated with primary rabbit anti-dopamine D_{2L} antibody (1:1000) at 4 °C overnight, followed by washing and incubation with polyclonal horseradish peroxidase-conjugated anti-rabbit IgG (Promega) (1:5000) for 2 h at room temperature. The immunoblots were developed by a chemiluminescence method and exposed to Kodak X-OMAT film.

2.7 Immunoprecipitation Method

Bovine striatal membrane protein (1 mg) was incubated with either anti-dopamine D_{2L} antibody or goat serum at 4 °C for 4 h followed by the addition of protein A sepharose beads for overnight incubation at 4 °C. Pellets collected after centrifugation (9,300 x g for 5 min) were washed once in 200 μl of Tris EDTA, followed by resuspension in receptor binding buffer. The samples were then centrifuged for 10 min at 16,600 x g, and the resultant pellet was solubilized in solubilization buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1% igepal, 0.5% sodium deoxychlorate, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF and a half tablet of protease inhibitor cocktail). The solubilized protein in the supernatant following centrifugation (16,600 x g for 20 min) was estimated using Bradford assay. The immunoprecipitated, solubilized bovine striatal samples were then incubated with 0.1 μM [^{125}I]-**1b**, in the absence and presence of 1 mM PLG, in a total volume of 1 ml for 1 h at 25 °C. The samples were cross-linked with UV light at a wavelength of 254nm for 5 min. Subsequently, they were centrifuged for 5 min at 10,000 rpm and the resulting pellet was washed once with Tris EDTA by centrifugation as described above. All samples were separated on a 12.5% SDS-PAGE and prepared for western blotting and autoradiography.

2.8 Data Analysis

Data was analyzed by one-way repeated measures of analysis of variance (ANOVA) using GraphPad Prism software, followed by Newman-Keuls post-hoc test. The data were analyzed for either one or two site populations of binding sites together with statistical analysis comparing the goodness of fit between one or two affinity state models. The intensities of autoradiographs were analyzed using ImageJ 1.40g software (National Institutes of Health, Bethesda, MD, USA) and relative optical densities of the Western blots

were analyzed using Northern Eclipse 6.0 software (EMPIX Imaging, Mississauga, ON, Canada).

3. RESULTS

3.1 Photoaffinity labeling the PLG Binding Site with PLG Analogue [^{125}I]-1b Shows Specific Binding to the D_{2L} Receptor

Photoaffinity labeling of membranes with analogue [^{125}I]-**1b** revealed specific binding to a 51 kDa protein in bovine striatal membranes (Fig 2A and 2B**, CTL lane**). Nonspecific labeling in striatal membrane was assessed by measuring the changes in [^{125}I]-**1b** binding in the presence of either **1a** (1 mM, Fig 2A) or PLG (10 μM and 100μM, Fig 2B). In the presence of either **1a** or PLG, the incorporation of [^{125}I]-**1b** was significantly ($P<0.001$) reduced as displayed by the signal strength. In the presence of PLG there was up to 60% displacement of [^{125}I]-**1b** radiolabeling of the 51 kDa protein. In addition to using autoradiography to indicate the specificity of **1b** for the PLG binding site, a competition curve was also generated with 2 nM of [^{125}I]-**1b** and increasing concentrations of PLG **(**Fig 3**).** This generated a one-site curve with an IC_{50} of 47 nM.

The dopamine D_{2L} receptor was successfully immunoprecipitated with a protein specific antibody, as confirmed by western blotting, which displays a band at 51 kDa for the bovine striatal membrane sample **(**Fig 4A**)**. Such a band was missing in the negative control of bovine striatal membrane immunoprecipitated with goat serum. The results outlined in Fig 4B display the autoradiograph after a 5 night exposure, which again shows a band at 51 kDa from the bovine striatal membrane sample immunoprecipitated with the anti-dopamine D_{2L} antibody and then incubated with [^{125}I]-**1b**. Similar to the western blot, no band was observed in the negative control sample. In Fig 4C, a fainter band was observed at 51 kDa for the sample treated with both [^{125}I]-**1b** and 1 mM PLG. These results are also displayed as a bar graph, which shows a significant ($P<0.05$) decrease in radiolabeling by [^{125}I]-**1b** in the presence of PLG (Fig 4D**)**.

3.2 2-Dimensional Gel Separation Further Confirms PLG's Binding Target as the D_{2L} Receptor

Given the heterogeneity of brain tissue, 2-dimensional (2D) gel electrophoresis was utilized in an attempt to better separate proteins, and determine if **1b** was specifically binding to the dopamine D_{2L} receptor. Coomassie staining of the 2D gel showed the presence of a number of separated proteins in bovine striatal tissue, including a protein aggregate at 51 kDa and 80kDa (Fig 5A). An autoradiograph of the 2D gel revealed a band at 51 kDa and approximate pI of 5.1 that was radiolabeled by [^{125}I]-**1b (**Fig 5B**)**. In addition, this 51 kDa protein was confirmed to be the dopamine D_{2L} receptor, as determined by immunodetection using a dopamine D_{2L} antibody (Fig 5C**)**.

4. DISCUSSION

In the present study, we have used the photoaffinity-labeling PLG peptidomimetics **1a** and **1b** to provide the first evidence for the interaction of PLG with a distinct protein in striatal tissue, suggested to be the dopamine D_{2L} receptor. Although these analogues were not as effective as the parent PAOPA at enhancing [^{3}H]-NPA binding to the dopamine D_{2L} receptor (Mishra et al., 1999), they were still more potent than PLG (Mishra et al., 1990). The increase in [^{3}H]-NPA binding in striatal membranes by compound **1b**, demonstrated that iodination of **1a** would not affect its actions on dopamine D_{2L} receptor modulation.

On the basis of the above pharmacological characteristics, [^{125}I]-**1b** was used in photoaffinity-labeling experiments to identify proteins that interact with PLG. With a 1D

gel, **1b** was shown to specifically bind to a protein in the bovine striatum with a molecular weight of 51 kDa **(**Fig 2**)**. The autoradiograph confirms that PLG competes with **1b** for its incorporation into the binding sites due to the reduction in signal strength with increasing concentrations of PLG. The specificity of **1b** binding to the PLG binding site was further confirmed by competition receptor binding assays **(**Fig 3**).** The competition curve suggests a single binding site with an IC_{50} of 47 nM. These results compliment the single radiolabeled band observed at 51 kDa upon photolysis of [^{125}I]-**1b** with striatal membranes.

With strong support of **1b** being incorporated into the PLG binding site, 2D gels were used in an attempt to better separate and isolate the protein with the binding site. 2D gels analyzed by coomassie staining **(**Fig 5A**)**, autoradiograph **(**Fig 5B**)** and immunoblotting with dopamine D_{2L} specific antibody **(**Fig 5C**)**, all confirmed the labeling of a band at 51 kDa thought to be the dopamine D_{2L}. The mass of proteins at a higher molecular weight (~80 kDa) observed in the 2D gels are likely an aggregation of hydrophobic proteins that are often times difficult to maintain in a soluble form (Bjellqvist et al., 1982;Chevallet et al., 1998;Molloy et al., 1998).

Autoradiography and immunoblotting with a dopamine D_{2L} specific antibody of both 1D and 2D gels showed detection of the protein at 51 kDa that corresponds to the molecular weight of the dopamine D_{2L} receptor. Further support of **1b** binding to the dopamine D_{2L} receptor is provided by western blotting and autoradiography of immunoprecipated dopamine D_{2L} receptors from bovine striatal membranes **(**Fig 4**)**. Autoradiographs of striatal membranes immunoprecipitated with dopamine D_{2L} antibody and subsequently treated with [^{125}I]-**1b** also showed a band at 51 kDa, indicating that **1b** is specifically binding to the immunoprecipitated dopamine D_{2L} receptor **(**Fig 4B**)**. Western blot analysis definitively indicated that the radiolabeled **1b** immunoprecipitate was indeed the dopamine D_{2L} receptor **(**Fig 4A**)**. Evidence provided herein, not only shows that **1b** binds to the dopamine D_{2L} receptor, but also shows that PLG competes with **1b** for the same binding site on the dopamine D_{2L} receptor. Consequently this competition results in less **1b** binding and thus a fainter radioactive band in comparison to the sample only treated with **1b (**Fig 4C and D).

Previous results have shown that PLG requires the dopamine D_{2L} receptor to be coupled to the G_i protein in order to have its modulatory effect (Verma et al., 2005). The importance of the dopamine D_{2L} receptor/G_i-protein complex is indicative of the possible site of interaction with PLG by which this endogenous neuropeptide may be modulating and increasing the number and affinity of the dopamine D_{2L} receptors in the high affinity state. Since the present work indicates that PLG is acting at a distinct site on the dopamine D_{2L} receptor, it is also possible that PLG is acting as an allosteric ligand. The proposed conformational change is frequently synonymous with the term allosteric modulation because allosteric modulators are described as modulators that occupy sites other than the “primary” site of ligand binding, the orthosteric site. There is currently an increasing body of evidence showing that many receptors of the GPCR superfamily possess extracellular binding sites. Specifically, both dopamine D_2 and -D_4 receptors have been shown to be allosterically modulated by amiloride and zinc (May et al., 2004).

As an allosteric modulator, PLG would be expected to modify the receptor conformation to cause a change in the agonist binding affinity to the dopamine D_2 receptor, a characteristic of allosteric modulators as defined by Christopoulos et al. and Christopoulos and Kenakin (Christopoulos et al., 2004; Christopoulos and Kenakin, 2002). PLG displays no effect in the absence of orthosteric ligands, therefore the normal spatial and temporal pattern of physiological signal transduction and termination is preserved. As is often the case with an allosteric modulator, PLG is only able to modify the pre-existing signaling pattern, as shown by this study as well as all previous studies involving PLG and its analogues (Chiu et al.,

1983; Mishra et al., 1999; Srivastava et al., 1988). Furthermore, toxic effects from modulators such as PLG are rare since there is a 'ceiling' to their effects and even a decreased effect once a compound-specific concentration has been passed (Raghavan et al., 2009).

In conclusion, we report that the PLG analogue **1b** labels a potential site for PLG binding, which is most likely the dopamine D_{2L} receptor, as established by autoradiography, immunoblotting, immunoprecipitation, and immunocytochemistry. Our findings are fundamental to the nature of PLG binding and signal transduction and offer insight into the mechanism of action for PLG. The results of this study will allow for future focused research with respect to binding parameters and greater sensitivity in an attempt to fully elucidate the mechanistic action of PLG, and its interaction with the dopamine D_{2L} receptor.

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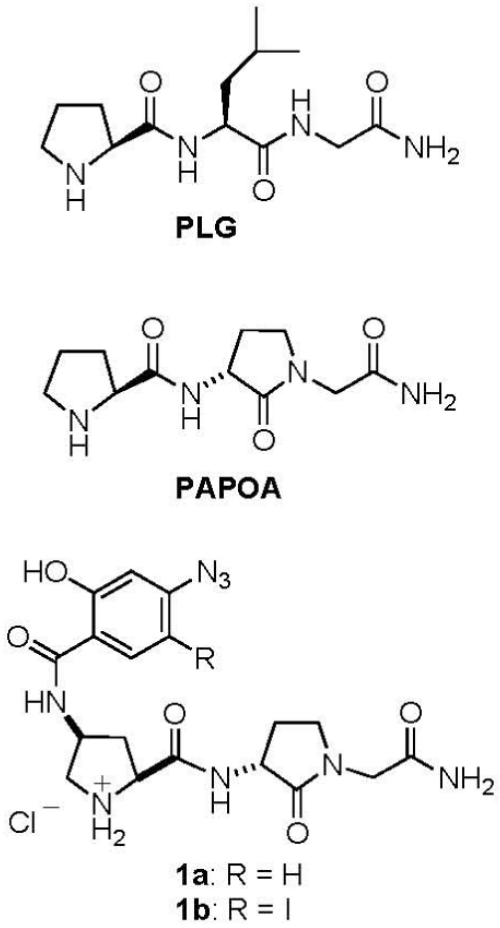

Fig 1. Structures of chemical compounds

Structures of L-prolyl-L-leucyl-glycinamide (PLG), the PLG peptidomimetic PAOPA, the photoaffinity-labeling compound 3*R*-[4(*S*)-(4-azido-2-hydroxy-benzoyl)amino-2(*S*)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1a**) and the photoaffinity-labeling compound 3(R)-[(4(S)-(4-azido-2-hydroxy-5-iodo-benzoyl)amino-2(S)-pyrrolidinylcarbonyl)amino]-2-oxo-1-pyrrolidineacetamide hydrochloride (**1b)**.

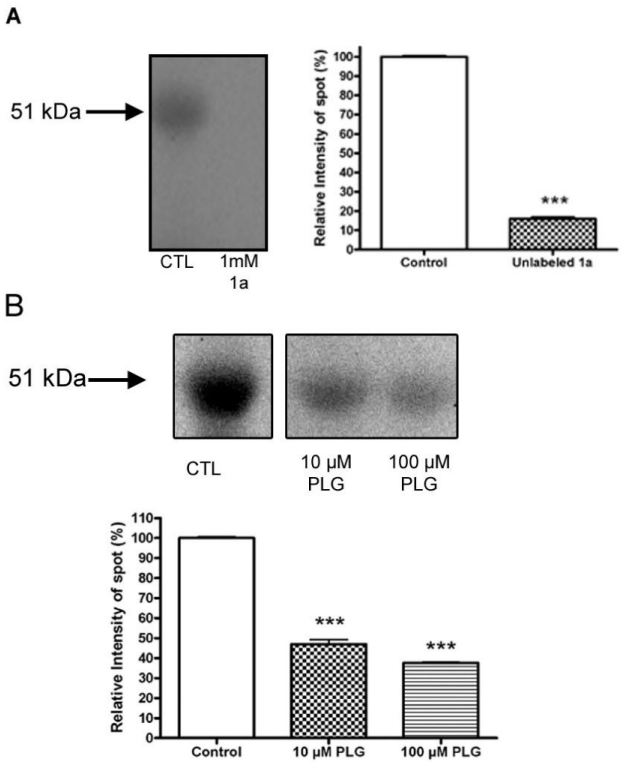

Fig 2. Displacement of photoaffinity-labeling PLG peptidomimetic 1b by peptidomimetic 1a and PLG in bovine striatal membranes

(A) Photolabeling of proteins by the PLG analogue **1b** (1 nM) was determined using 200 μg of homogenized bovine striatal membranes (lane CTL). Nonspecific labeling by analogue **1b** was determined by displacement with 1 mM **1a**. The relative intensity (%) of autoradiograph signal was quantified and presented as a bar graph. The data was analyzed by Student's t-test (*** significance $P<0.001$ from control value) **(B)** Displacement of PLG analogue **1b** (1 nM) was performed by incubation with 10 μM or 100 μM of PLG. The relative intensity (%) of autoradiograph signal was quantified and presented as a bar graph. The data was analyzed by a one way analysis of variance followed by Tukey's post-hoc test: (***) significantly different ($P<0.001$) from control value.

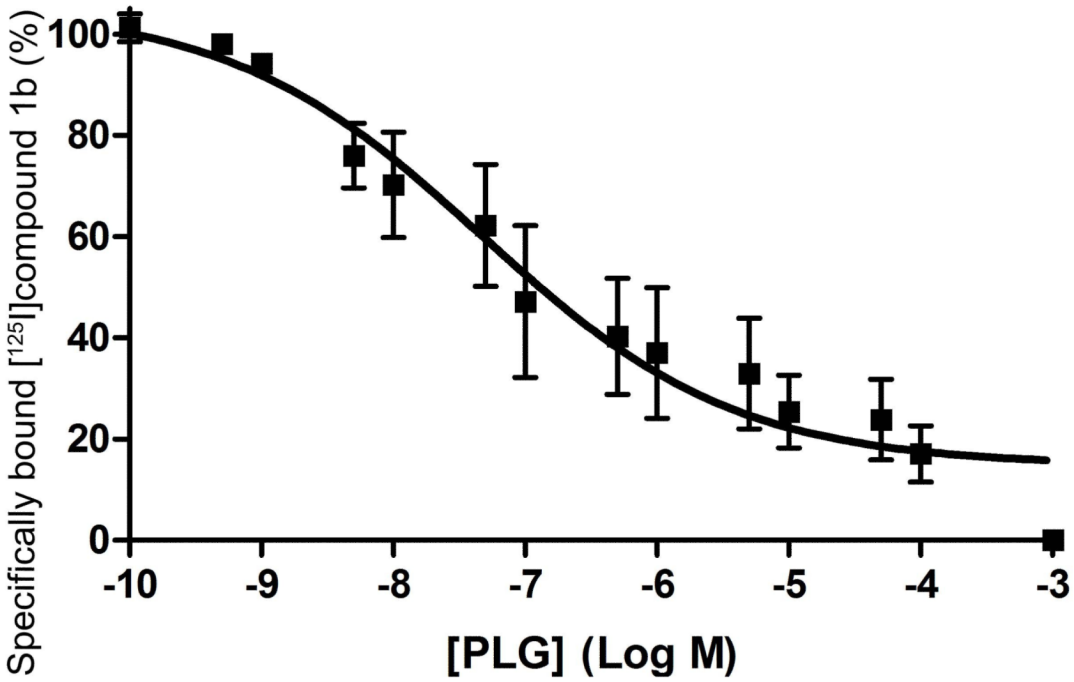

Fig 3. Competition curve of photoaffinity-labeling PLG peptidomimetic 1b and PLG

Competition curve of **1b** and PLG generated from the mean (±S.E.M.) of 3 experiments of triplicates. Bovine striatal membranes were incubated with 2 nM of [^{125}]-**1b** and increasing concentrations of PLG.

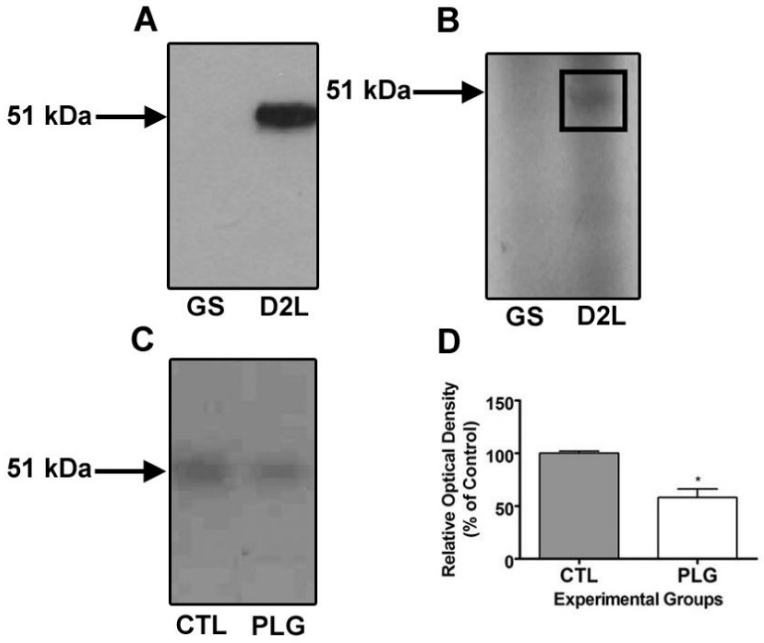

Fig 4. Identification of protein labeled with photoaffinity-labeling PLG peptidomimetic 1b using 1D SDS-PAGE

(A) Western blot showing a band at approximately 51 kDa for a bovine striatal membrane sample immunoprecipitated with the dopamine D_{2L} antibody (lane D2L). There is no band for the bovine membrane sample immunoprecipitated with goat serum (lane GS). **(B)** Autoradiograph after a 5 night exposure showing a band at approximately 51 kDa for the bovine membranes sample immuoprecipitated with dopamine D_{2L} antibody and post treated with **1b**. **(C)** Autoradiograph displaying bands for membrane sample immunoprecipiated with dopamine D_{2L} antibody and subsequently treated with **1b** in the absence and presence of 1 mM PLG. **(D)** Bar graph displaying the optical density percentage of bands from Figure 4C (*P<0.05).

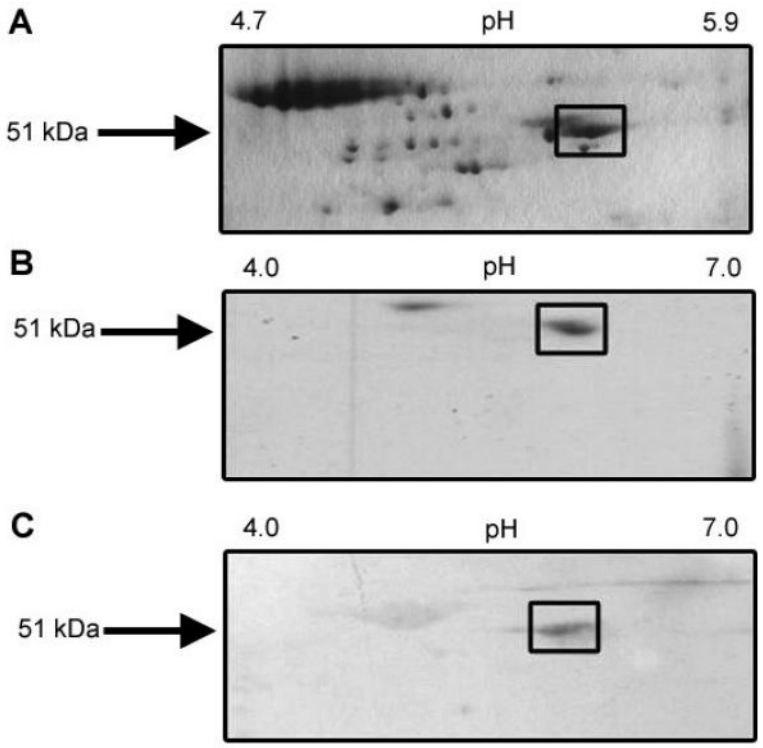

Fig 5. Identification of protein labeled with photoaffinity-labeling PLG peptidomimetic 1b using 2D SDS-PAGE

(A) Coomassie stained 2D SDS-PAGE of IPG strip with a ranging pI of 4.7-5.9, showing the protein of interest located at 51kDa. **(B)** Displays the protein labeled by **1b** in bovine striatal membranes ranging between the pI of 4-7. **(C)** Shows the western blot of a 2D gel with bovine striatal membrane separated on a 4-7 IPG strip. Immunoblots were prepared using dopamine D_{2L} antibody (dilution of 1:1000) after blocking with 10% milk. All blots and autoradiographs detected the same protein (indicated by the box) located at 51 kDa and pI 5.1, determined by superimposing blots with autoradiographs.