JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Central GPR109A activation Mediates Glutamate-Dependent Pressor Response in Conscious Rats

Samar Rezq and Abdel A. Abdel-Rahman,

Department of Pharmacology, School of Medicine, East Carolina University, NC, U.S.A.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

a) Running title: RVLM GPR109A Mediates Glutamate-Dependent Pressor Response

b) Corresponding author: Abdel A Abdel-Rahman, Ph.D

Department of Pharmacology, The Brody School of Medicine, East Carolina University, Greenville, NC 27834. Tel 252-744-3470; Fax: 252-744-3203; Email: abdelrahmana@ecu.edu

c) Number of text pages: 15

Number of tables: 1

Number of figures: 9

Number of references: 72

Word count:

Abstract: 247

Introduction: 542

Discussion: 1465

d) Abbreviations:

ACSF (artificial cerebrospinal fluid); ANOVA (Analysis Of Variance); AUC (area under the curve); BP (Blood pressure); DAF-FM Diacetate (difluorofluorescein diacetate); DCFH-DA (2′,7′-Dichlorofluorescein diacetate); DHE (dihydroethidium); G protein coupled receptor 109A (GPR109A); HR (heart rate); IF (immunofluorescence); IsoNA (iso-nicotinic acid); NA (nicotinic acid); NMDAR (NMDA receptor); nNOS (neuronal nitric oxide synthase); NO (nitric oxide); PC12 cells (Rat pheochromocytoma cell line); ROS (reactive oxygen species); RVLM (the rostral ventrolateral medulla); SD (Sprague Dawley); TH (tyrosine hydroxylase); WB (western blot).

e) Recommended section assignment: Cardiovascular

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

G protein-coupled receptor 109A (GPR109A) activation by its ligand nicotinic acid (NA) in immune cells increases Ca^{2+} levels, and Ca^{2+} induces glutamate release, and oxidative stress in central blood pressure (BP) regulating nuclei e.g. the rostral ventrolateral medulla (RVLM) leading to sympathoexcitation. Despite NA ability to reach the brain, the expression and function of its receptor GPR109A in the RVLM remains unknown. We hypothesized that NA activation of RVLM GPR109A causes Ca^{2+} dependent L-glutamate release, and subsequently increases neuronal oxidative stress, sympathetic activity, and BP. To test this hypothesis, we adopted a multilevel approach, which included pharmacological in vivo studies along with ex vivo and in vitro molecular studies in PC12 cells (exhibit neuronal phenotype). We present the first evidence for GPR109A expression in the RVLM and in PC12 cells. Next, we showed that RVLM GPR109A activation (NA) caused pressor and bradycardic responses in conscious rats. The resemblance of these responses to those caused by intra-RVLM glutamate, and their attenuation by NMDA receptor (NMDAR) blockade (2-amino-5-phosphonopentanoic acid; AP5) and enhancement by L-glutamate uptake inhibition (L-trans-Pyrrolidine-2,4-dicarboxylic acid; PDC) supported our hypothesis. NA increased Ca^{2+}, glutamate, nitric oxide (NO) and reactive oxygen species (ROS) levels in PC12 cells and increased RVLM ROS levels. The inactive NA analog iso-nicotinic acid (IsoNA) failed to replicate the cardiovascular and biochemical effects of NA. Further, GPR109A knockdown (siRNA) abrogated the biochemical effects of NA in PC12 cells. These novel findings yield new insight into the role of RVLM GPR109A in central BP control.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

The recently identified G protein coupled receptor 109A (GPR109A) is now recognized as the molecular target that mediates the long established anti-hyperlipidemic effect of nicotinic acid (NA) (Canner et al., 1986; Carlson, 2005). The most common side effect of NA is the prostaglandins-dependent flushing reaction (Andersson et al., 1977; Morrow et al., 1989). The latter involves intracellular Ca^{2+} elevation and subsequent activation of phospholipase A2 (Lin et al., 1992; Benyo et al., 2006). In the CNS, higher intracellular Ca^{2+} levels enhance neurotransmitters release, including glutamate (Kish and Ueda, 1991; Berridge, 1998; Sudhof, 2004).

GPR109A is widely expressed in white and brown adipose tissue, keratinocytes and various immune cells including monocytes, macrophages, neutrophils and dendritic cells including Langerhans cells (Soga et al., 2003; Tunaru et al., 2003; Wise et al., 2003; Hanson et al., 2010), and likely in microglia (Penberthy, 2009). Despite NA ability to enter the brain (Spector, 1979), there are no reports on the expression or function of GPR109A in cardiovascular regulating nuclei including the RVLM. The latter sends excitatory input to the sympathetic preganglionic neurons, and regulate the sympathetic nervous system and peripheral cardiovascular activity (Ciriello et al., 1986; Chapp et al., 2014).

Realizing that NA activation increases intracellular Ca^{2+} in other cell types (Benyo et al., 2006), it is likely that access of NA to central GPR109A might produce a similar effect and triggers L-glutamate release in the RVLM, which is Ca^{2+}-dependent (Tingley and Arneric, 1990). L-glutamate activation of fast-acting ionotropic receptors, including NMDA and AMPA receptors increases intracellular Na^{+} and Ca^{2+} as well as nerve firing (Dingledine et al., 1999). While the L-glutamate activation of the central ionotropic or the metabotropic receptors (Gabor and Leenen, 2012) causes sympathoexcitation and a pressor response (Ito et al., 2003), the NMDA receptor (NMDAR)-mediated fast and brief pressor response prevails in the RVLM (Kubo et al., 1993). Further,

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

elevations in Ca^{2+} (Lipton and Rosenberg, 1994) and L-glutamate (Albrecht et al., 2010) levels lead to oxidative stress, and the occurrence of the latter in the RVLM leads to sympathoexcitation and pressor response (Nishihara et al., 2012).

In the present study, we tested the hypothesis that Ca^{2+}-dependent L-glutamate release and oxidative stress, triggered by NA activation of RVLM GPR109A, causes sympathoexcitation and a pressor response. We adopted a multilevel approach, which included pharmacological cardiovascular studies in conscious rats complemented with ex vivo and in vitro molecular studies. We conducted the detailed molecular studies in vitro, because the tissue scarcity and the rapid onset of the NA-evoked pressor response precluded conducting detailed ex vivo studies on RVLM tissue. Importantly, differentiated PC12 cells, used in these studies, exhibit a sympathetic-neuron-like phenotype (Greene, 1978; Penugonda et al., 2005). Following the first demonstration of GPR109A expression in the RVLM and in PC12 cells, we investigated the effects of intra-RVLM NA, or its inactive isomer iso-nicotinic acid (IsoNA), on BP and heart rate (HR) in conscious rats. Additional in vivo studies were conducted in the presence of NMDAR blockade (2-amino-5-phosphonopentanoic acid; AP5), non-NMDAR (AMPA/Kainate) blockade (6-cyano-7-nitroquinoxaline-2,3-dione; CNQX) or L-glutamate uptake inhibition (L-trans-Pyrrolidine-2,4-dicarboxylic acid; PDC) in the RVLM. The effects of these pharmacological interventions or GPR109A knockdown (siRNA) on the GPR109A (NA)-mediated biochemical responses were also investigated in PC12 cells to complement, and to mechanistically explain, the in vivo findings.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Materials and Methods

Animal Preparation.

Male Sprague Dawley (SD) rats (320–370 g; Charles River Laboratories, Raleigh, NC) were used in this study. All rats were housed two per cage in a room with a controlled environment at a constant temperature of 23 ± 1°C, humidity of 50% ± 10%, and a 12-hour light/dark cycle. Food (Prolab Rodent Chow, Prolab RMH 3000; Granville Milling, Creedmoor, NC) and water were provided ad libitum. All surgical, postoperative care, and experimental procedures were performed in accordance with, and approved by the Institutional Animal Care and Use Committee and in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute for Laboratory Animal Research, 2011). Surgical procedures, BP and HR measurements were performed as reported in our recent study (Penumarti and Abdel-Rahman, 2014), and as detailed in the Supplemental Material.

Cell Culture.

Rat pheochromocytoma cell line (PC12 cells) was purchased from ATCC (Rockville, MD). Cells were grown in F12k medium supplemented with horse serum (15%), fetal bovine serum (2.5%), penicillin (100U/ml), and streptomycin (100U/ml) at 37°C in a humidified incubator with 95% air and 5% CO_2. The cells were cultured on corning cell bind flasks for proper adherence followed by treatment with nerve growth factor (50ng/ml) till completely differentiated to the neuronal phenotype according to the protocol of the supplier. Passage was done every 3-5 days when cells reach confluence as in our previous studies (Zhang et al., 2001).

Western Blot Analysis and Immunofluorescence.

We followed the protocols described in the online supplemental material and in our previous studies (Ibrahim and Abdel-Rahman, 2012; Penumarti and Abdel-Rahman, 2014).

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

$(Ca^{2+})_i$, Glutamate, and NO Measurements.

$(Ca^{2+})_i$ was measured in PC12 cells using cell permeant fura-2 AM (1 μM) (Molecular Probes, Invitrogen) as detailed in the Supplemental Material. For L-glutamate measurement, Amplex Red kit was used (Molecular Probes, Invitrogen) following the manufacturer instructions and reported studies (Medina-Ceja et al., 2015) as provided in the Supplemental Material. NO was measured using 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM Diacetate) (Molecular Probes, Grand Island, NY) according to the manufacturer instructions and reported studies (Cortese-Krott et al., 2012) as described under methods in the online supplemental material.

Measurement of Oxidative Stress.

We used 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Grand Island, NY), a general detector of oxidative species, to measure oxidative stress in PC12 cells and in RVLM specimens taken from animals that received NA or its vehicle. Further, the dihydroethidium (DHE) staining method, which is more superoxide anion selective (Bindokas et al., 1996; Robinson et al., 2006) was used for ROS measurements in RVLM sections of animals that received the different treatments described below. The methods and protocols used are detailed in our previous studies (Penumarti and Abdel-Rahman, 2014) and described in the online supplemental material.

siRNA Knockdown of GPR109A in PC12 Cells.

PC12 cells in suspension (150,000 cells/ml) were transfected with 2.5 μg GPR109A siRNA Flexitube Gene Solution or Allstars negative control siRNA (Qiagen, KY) using 12 μl HiPerFect transfection reagent (Qiagen, KY) in 6 wells plates for 24 h according to the manufacturer's

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

protocol. A substantial (≈70%) reduction in GPR109A expression (western blot) validated the adopted siRNA knockdown strategy.

Experimental Design and Protocols

Experiment 1: Expression of GPR109A in the RVLM and PC12 Cells.

The objective of this experiment was to determine if GPR109A is expressed in the RVLM and in PC12 cells, and its spatial distribution in the RVLM relative to different cell types, particularly tyrosine hydroxylase (TH) immunoreactive (ir) neurons, which modulate the sympathetic activity and BP (Guyenet, 2006; Kumagai et al., 2012). First, western blot (WB) analysis was conducted to detect GPR109A in PC12 cell lysate, and in punches taken from the RVLM of naïve rats (n = 3). Tissues rich in (spleen), or devoid of (liver) GPR109A (Soudijn et al., 2007) were used as positive and negative controls, respectively, in these studies. Second, dual labeled immunofluorescence (IF) was conducted in post-fixed sections from rat frozen brains and PC12 cells. Neuronal and glial cell markers including the neuronal marker, NeuN, the astrocyte marker, GFAP, and the microglia marker, CD11b/c were used to investigate the spatial distribution of GPR109A in different cell types in the RVLM. Further the expression of GPR109A relative to TH-ir neurons was investigated by using an anti-tyrosine hydroxylase antibody. We followed methods described in our previous studies (Ibrahim and Abdel-Rahman, 2011; Penumarti and Abdel-Rahman, 2014) and detailed in the online supplemental material.

Experiment 2: Functional Role of RVLM GPR109A in BP and HR Regulation.

As there are no reported studies on intra-RVLM NA, a preliminary study was conducted to determine the optimal NA dose to be used throughout the study based on estimated penetration of systemic NA into the CNS (Spector, 1979). Two groups of conscious unrestrained SD rats (n = 4-7), instrumented for BP and HR measurements and RVLM microinjections as detailed under methods, received intra-RVLM NA (5, 10 and 20 µg/animal) or the vehicle, artificial

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

cerebrospinal fluid (ACSF). The 20 µg NA dose, which produced consistent pressor response, was used in subsequent studies. There are currently no GPR109A antagonists. Therefore, to ensure that the NA-evoked pressor response was GPR109A mediated, the same dose (20 µg/animal) of the NA inactive isomer, IsoNA, was microinjected into the RVLM of a third group of rats (*n* = 6).

Experiment 3: Effect of NMDAR, non-NMDAR Blockade or L-glutamate Uptake Inhibition on the Cardiovascular Effects of NA.

The role of L-glutamate in NA-induced pressor response was investigated using different pharmacological interventions in conscious male SD rats instrumented for measurements of BP and HR and RVLM microinjections as detailed under *methods*. Four groups of rats (*n* = 5-7) received the NMDAR blocker, AP5 (2 nmol/100nl) (Zhang and Abdel-Rahman, 2002), the non-NMDAR blocker, CNQX (200 pmol) (Wang et al., 2007), the glutamate uptake inhibitor, PDC (1 µM) (Montiel et al., 2005) or ACSF 10 min before NA (20 µg/animal) microinjection.

Experiment 4: GPR109A-dependent Biochemical Effects of NA.

The objective of this experiment was to obtain direct biochemical evidence that GPR109A mediates elevations in L-glutamate release, intracellular Ca^{2+} and reactive oxygen species (ROS), which are implicated in sympathoexcitation and BP elevation (Lipton and Rosenberg, 1994; Ito et al., 2003; Nishihara et al., 2012). PC12 cells, used as surrogates of RVLM neurons, were incubated with NA (10 µM, 100 µM or 1mM) in the absence or the presence of the pharmacological interventions used in vivo (experiment 3). This model system permitted rapid (0.5-1 min) biochemical measurements after NA addition, a timeframe consistent with the rapid onset of the pressor response caused by intra-RVLM NA. The dependence of the biochemical effects of NA on GPR109A activation was verified in PC12 cells by: (i) replacing NA with its inactive isomer, IsoNA (Maciejewski-Lenoir et al., 2006) (1 mM); (ii) the addition of NA (1 mM) to

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

PC12 following GPR109A knockdown with siRNA (see methods). The pharmacological interventions incubated with PC12 cells in the absence or the presence of NA (1 mM) included: (i) L-glutamate in a concentration (100 nM) that was achieved 1 min after NA (1 mM) addition to PC12 (preliminary experiment); (ii) the glutamate uptake inhibitor, PDC (0.1 mM) for 1 h (Pepponi et al., 2009); (iii) the NMDAR blocker, AP5 (100 μM) for 10 min (Reigada et al., 2006). For ex vivo ROS measurements, brain tissues were collected from animals (n = 4–5 rats) that received the different treatments following euthanasia with a lethal dose of pentobarbital sodium (>100 mg/kg) at the end of BP recording period. Fresh unfixed brainstem sections (20 μm) sections were taken followed by collecting micropunches from the injected RVLM side using a 0.75 micropunch instrument (Stoelting Co., Wood Dale, IL), using the following coordinates 12.6 to -11.8 mm relative to bregma as in reported studies including ours (Paxinos et al., 1980; Ibrahim and Abdel-Rahman, 2015).

Drugs.

NA, L-glutamate, and PDC were purchased from Sigma-Aldrich (St. Louis, MO). DL-AP5 and CNQX were purchased from Tocris Bioscience (Ellisville, MO). Sterile saline was purchased from B. Braun Medical (Irvine, CA). IsoNA acid was purchased from TCI America (Portland, OR).

Statistical Analysis.

In vitro data are representative of three independent experiments. Animal studies data represents n of 3-7. Data are expressed as mean ± standard error of mean (S.E.M). Analysis of Variance (ANOVA) or repeated-measures analysis of variance followed by Bonferroni's post hoc test and Student's t-test were carried out using GraphPad Prism to state differences between groups.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Results

GPR109A Co-expression with Tyrosine Hydroxylase in the RVLM and in PC12 Cells.

WB analysis revealed and verified the expression GPR109A in the RVLM and PC12 cells, when compared to tissues rich in (spleen) or devoid of (liver) GPR109A expression (Fig. 1A). Further, dual-labeled IF findings in PC12 cells showed co-expression of GPR109A and TH (Fig. 1B). Cell type specific labeling (Fig. 2 A-D), and quantitative comparisons of GPR109A expression in different cell types revealed much greater percentage (approx. 70%) of the counted neuronal than non-neuronal (astrocytes and microglia) (30%) cells of the RVLM expressed GPR109A. Further, the majority (>90%) of the RVLM TH-ir neurons also expressed GPR109A (Fig. 2E).

Activation of RVLM GPR109A Causes Glutamate-like Pressor Response and Neuronal Oxidative Stress.

BP and HR values before intra-RVLM microinjection of NA in the absence or presence of pharmacologic interventions, indicated below, were not significantly different (Table 1). Intra-RVLM NA (5, 10, 20 µg) caused dose-dependent pressor response and bradycardia in conscious rats (Fig. 3). These cardiovascular responses resembled the responses elicited by intra-RVLM L-glutamate (1 nmol; 169 ng) (Fig. 3A). Importantly, the inactive NA isomer, IsoNA (20 µg), failed to influence BP or HR (Fig. 4, A and B). Ex vivo studies revealed higher ROS levels in NA (20 µg)-treated RVLM, but not following the same dose of IsoNA (Fig. 5).

Selective NMDAR Blockade and Glutamate Uptake Inhibition Abrogates and Enhances, Respectively, the Intra-RVLM NA-Evoked Oxidative Stress and Pressor Response.

Prior RVLM NMDAR (AP5; 2 nmol), but not non-NMDAR (CNQX; 200 pmol) blockade abrogated ($P < 0.05$) the pressor and bradycardic responses (Fig. 4, C, D and E), and abolished the increase in ROS (Fig. 5B) caused by subsequent intra-RVLM NA (20 µg). On the other hand, prior L-glutamate uptake inhibition with intra-RVLM PDC (1 µM) significantly ($P < 0.05$)

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

increased the magnitude and duration (presented as area under the curve, AUC) of the pressor response (Fig. 4, C and E) and exacerbated the elevation in RVLM ROS (Fig. 5B) caused by subsequent intra-RVLM NA (20 µg) microinjection. None of these intervention changed BP or HR from the corresponding baseline values (Table 1).

GPR109A Activation Increases L-glutamate, Ca^{2+} and NO levels and Produces NMDAR-Dependent Oxidative Stress in PC12 cells.

The rapid onset (approx. 1 min) of the pressor response caused by intra-RVLM NA precluded measurements of RVLM L-glutamate, Ca^{2+} and NO levels during this timeframe. Therefore, such measurements were made in differentiated PC12 cells, which exhibit neuronal phenotype and express GPR109A (Fig. 1, A and B). NA (100 µM or 1mM) significantly ($P < 0.05$) increased Ca^{2+} (Fig. 6A), L-glutamate (Fig. 6B), and NO (Fig. 6C) levels 30-60 sec after NA addition to the incubation medium. Pre-incubating PC12 cells with the L-glutamate uptake inhibitor PDC (0.1 mM/1 h) significantly ($P < 0.05$) enhanced NA-evoked glutamate release (Fig. 6B). These biochemical responses were associated with significant ($P < 0.05$) increases in ROS levels (Fig. 7A). Notably, L-glutamate, in a concentration (100 nM), detected after NA (1 mM) addition to incubation medium (preliminary), significantly ($P <0.05$) increased ROS level (Fig. 7B). Similar to ex vivo findings on RVLM (Fig. 5), the NA-induced oxidative stress was circumvented by prior NMDAR blockade with AP5 (100 µM) (Fig. 7B). Further, the inactive NA isomer, IsoNA (1 mM), had no effect on Ca^{2+}, glutamate, NO (Fig. 6, A, B and C) or ROS (Fig. 7A) levels.

GPR109A Knockdown Abolishes NA Induced Ca^{2+} and Glutamate Release in PC12 Cells.

There are no available GPR109A antagonists. Therefore, we used siRNA knockdown strategy to validate the involvement of GPR109A in the NA-evoked biochemical responses in PC12 cells, described above. The GPR109A targeting siRNA significantly ($P < 0.05$) reduced GPR109A expression (Fig. 8A). In PC12 cells pretreated with the negative siRNA control

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

(scrambled siRNA), NA (1 mM) significantly ($P < 0.05$) increased Ca^{+2} (Fig. 8B) and glutamate (Fig. 8C) levels. However, in PC12 cells, which exhibited significant ($P < 0.05$) siRNA-induced reduction in GPR109A expression (Fig. 8A), NA (1 mM) failed to produce these biochemical responses (Fig. 8, B and C).

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In this study we tested the hypothesis that NA activation of RVLM GPR109A induces L-glutamate (NMDAR)-dependent oxidative stress and sympathoexcitation/pressor response. We focused on the RVLM because it mediates L-glutamate-dependent increases in sympathetic activity and BP, and tonically regulates the peripheral vascular activity (Ciriello et al., 1986; Bazil and Gordon, 1993; Chapp et al., 2014).

Our most important findings are: (i) the majority of RVLM neurons, and particularly the TH-ir neurons, express GPR109A; (ii) intra-RVLM injection of the GPR109A agonist NA, but not its inactive isomer (IsoNA) caused elevations in RVLM oxidative stress and BP, which resembled glutamate-evoked responses; (iii) NMDAR blockade (AP5) and L-glutamate uptake inhibition (PDC) abrogated and exacerbated, respectively, the NA-evoked increases in BP and RVLM oxidative stress; (iv) NA, but not IsoNA, increased Ca^{2+}, NO, glutamate, and ROS levels in PC12 cells; (v) GPR109A knockdown (siRNA) abrogated the NA-evoked biochemical responses in PC12 cells.

GPR109A, which serves as a receptor for NA (Soga et al., 2003; Tunaru et al., 2003), is expressed in adipose tissue and immune cells (Soga et al., 2003; Maciejewski-Lenoir et al., 2006; Penberthy, 2009). Under normal physiological conditions, the levels of the endogenous agonist (β-hydroxybutyrate) or exogenous agonist (NA), when taken as a vitamin, might be lower than the μM-mM concentration range needed to activate GPR109A (Taggart et al., 2005). However, when used in much higher doses as anti-hyperlipidemic drug (Brown et al., 2001; Whitney et al., 2005), such NA concentrations might be achieved. Further, despite evidence that NA is transported into the brain in appreciable concentrations following systemic administration (Spector, 1979), there are no reports on GPR109A expression or function in neuronal structures, which regulates sympathetic outflow and BP (e.g. RVLM). Here, we present the first

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

evidence that GPR109A is expressed in RVLM neurons, including the TH expressing neurons (Fig. 2, A and B); the latter modulate the sympathetic activity and BP (Guyenet, 2006; Kumagai et al., 2012). These findings, which infer a cardiovascular role for GPR109A, were confirmed by observing dose-related increases in BP following intra-RVLM NA microinjection in conscious rats (Fig. 3B). The rapid onset of the NA-induced pressor response along with the associated bradycardia were reminiscent of cardiovascular responses elicited by L-glutamate microinjection into the same neuronal pool observed in a separate group of rats (Fig. 3A), and in reported studies including ours (Bachelard et al., 1990; Mao and Abdel-Rahman, 1994). While the rapid onset of the pressor response (Fig. 3A) precluded investigation of NA effect on RVLM L-glutamate release, we adopted pharmacological approaches in vivo and direct biochemical measurements in PC12 cells to support L-glutamate involvement in the NA-evoked cardiovascular responses. It is imperative, nonetheless, to note that these cardiovascular responses were mediated via NA activation of RVLM GPR109A because the inactive NA isomer, IsoNA, failed to produce similar cardiovascular effects (Fig. 4, A and B).

We hypothesized that NA activation of GPR109A in the RVLM leads to Ca^{2+}-dependent L-glutamate release because: (i) NA (10 µM-3 mM) mediates GPR109A dependent increase in intracellular Ca^{2+} levels in macrophages and epidermal Langerhans cells (Benyo et al., 2005; Benyo et al., 2006; Vanhorn et al., 2012; Gaidarov et al., 2013), (ii) Ca^{2+} triggers L-glutamate release (Kish and Ueda, 1991; Berridge, 1998; Sudhof, 2004), and (iii) elevated L-glutamate levels cause oxidative stress (Wang et al., 2013; Yang et al., 2014), sympathoexcitation (Chapp et al., 2014), and ultimately BP elevation (Iwata et al., 1987; Bazil and Gordon, 1993). Therefore, the RVLM GPR109A most likely mediates a glutamate-dependent sympathoexcitation. Although the origin of NA-induced glutamate release in the RVLM remains uncertain, the RVLM presympathetic neurons contribute, at least partly to this process because they exhibit the highest GPR109A level in the RVLM (Fig. 2E), and their axonal varicosities

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

contain the glutamate transporter VGLUT2, and release glutamate (DePuy et al., 2013). Our in vivo and ex vivo findings supported the L-glutamate hypothesis because local NMDAR blockade (AP5) abrogated, while glutamate uptake inhibition (PDC) exacerbated, the pressor response (Fig. 4) and RVLM oxidative stress (Fig. 5) caused by intra-RVLM NA.

Next, we leveraged the biological phenotype resemblance of the differentiated PC12 cells and RVLM neurons (Separovic et al., 1997; Zhang et al., 2001) to obtain direct evidence that NA activation of GPR109A leads to L-glutamate and Ca^{2+} release. However, in the absence of any reports on GPR109A expression in PC12 cells, it was important to determine if, similar to the RVLM neurons, the receptor is expressed, and is spatially associated with TH, in this cell line. Our WB and dual labeling IF (Fig. 1, A and B) confirmed our assumptions, and validated the use of differentiated PC12 cells as an appropriate model system for studying the biochemical events triggered by NA activation of GPR109A. We showed that NA causes increases in Ca^{2+} and L-glutamate levels in PC12 cells (Fig. 6, A and B) along with oxidative stress (Fig. 7A).

It was important to determine if the GPR109A-mediated increase in glutamate release (Fig. 6B) accounts for the oxidative stress (Fig. 7A) because the latter is implicated in sympathoexcitation as discussed above. Our findings support this view because: (i) NA (1 mM) and L-glutamate, in a concentration (100 nM) similar to that released by NA (1 mM), produced comparable increases in oxidative stress in PC12 cells (Fig. 7B); (ii) L-glutamate uptake inhibition augmented the NA-induced glutamate release (Fig. 6B). Although no GPR109A antagonists are currently available, these effects are most likely GPR109A-dependent because the inactive NA isomer IsoNA, whose microinjection into the RVLM had no effect on BP or RVLM redox state (Figs. 4 and 5), also failed to reproduce NA-evoked biochemical effects in PC12 cells (Figs. 6 and 7). The latter findings agree with reported studies in other model systems (Maciejewski-Lenoir et al., 2006; Gaidarov et al., 2013). Equally important, NA failed to

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

increase Ca^{2+} or glutamate levels in PC12 cells following siRNA-evoked GPR109A knockdown (Fig. 8), which provided direct evidence for GPR109A mediation of NA actions, and support the in vivo findings, discussed above.

It is important to comment on the role of the NMDAR in mediating the biochemical and pressor responses caused by NA activation of GPR109A. As discussed above, NA activation of GPR109A leads to the release of L-glutamate, which activates ionotropic and metabotropic glutamate receptors (Gabor and Leenen, 2012). The rapid onset of the NA-evoked pressor response is consistent with L-glutamate activation of the NMDAR ionotropic receptors in the RVLM (Kubo et al., 1993). This assumption was confirmed by the ability of the NMDAR blocker AP5 (Fig. 4C), but not the non-NMDAR blocker CNQX (Fig. 4E), to abrogate the NA-evoked pressor response as well as oxidative stress (Fig. 5B). Further, NMDAR activation by L-glutamate mediates a Ca^{2+}/calmodulin-dependent nNOS-derived NO generation and neurotoxicity (Garthwaite et al., 1988; Dawson et al., 1993). There is also evidence that NO is involved in glutamate-mediated neurotransmission in different brain areas (Southam et al., 1991; Montague et al., 1994) including the RVLM (Martins-Pinge et al., 1999). Additionally, the nNOS-derived NO in the RVLM causes sympathoexcitation in reported studies including ours (Martins-Pinge et al., 2007; Ibrahim and Abdel-Rahman, 2012). Therefore, It is likely that the increased levels of NO by NA (0.01-1mM) in PC12 cells (Fig. 6C) levels, which agrees with a reported finding in cultured endothelial cells treated with similar (0.5-2mM) NA concentrations (Huang et al., 2012), is probably due to the activation of NMDAR, via GPR109A mediated glutamate release. Notably, higher NO levels can contribute to RVLM oxidative stress via its interaction with elevated superoxide levels, detected by DHE staining in the RVLM of NA treated rats (Fig. 5B), to produce peroxynitrite radical (Beckman et al., 1990), which causes dose-dependent transient excitatory responses when injected into the RVLM (Zanzinger, 2002). Conversely, others reported that intra-RVLM injection of NO precursor or donor decreases

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

sympathetic nerve activity and BP (Kagiyama et al., 1997). Therefore, the possibility must be considered that the GPR109A-mediated increase in NO levels could either contribute to, or oppose, the pressor effect of intra-RVLM NA.

In conclusion, the present study presents the first evidence for GPR109A expression in the RVLM, and its mediation of sympathoexcitation and elevation of BP via glutamate/NMDAR-dependent mechanisms (Fig. 9). The complementary pharmacologic and biochemical findings clearly implicate the GPR109A in the observed responses. The present pharmacological findings might have clinical relevance in situations where high doses of NA are prescribed for the treatment of hyperlipidemia. Further, the present findings might have pathophysiological ramifications, particularly in hypertension for two reasons. First, there is a clear link between an exacerbated RVLM neuronal oxidative stress and the elevated BP in hypertension (Wu et al., 2014). Second, compared to normotensive controls, activation of RVLM NMDAR causes greater elevation in BP in spontaneously hypertensive rats (Lin et al., 1995). Collectively, the present findings, along with these reported studies, highlight central GPR109A as molecular target for the development of novel antihypertensive medications.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

The authors thank Kui Sun and Dr. Fanrong Yao for technical assistance.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Author Contributions

Participated in research design: Rezq, Abdel-Rahman.

Conducted experiments: Rezq.

Performed data analysis: Rezq.

Contributed to the writing of the manuscript: Rezq, Abdel-Rahman.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

References

Albrecht P, Lewerenz J, Dittmer S, Noack R, Maher P and Methner A (2010) Mechanisms of oxidative glutamate toxicity: the glutamate/cystine antiporter system xc- as a neuroprotective drug target. *CNS & neurological disorders drug targets* **9**:373-382.

Andersson RG, Aberg G, Brattsand R, Ericsson E and Lundholm L (1977) Studies on the mechanism of flush induced by nicotinic acid. *Acta pharmacologica et toxicologica* **41**:1-10.

Bachelard H, Gardiner SM and Bennett T (1990) Cardiovascular responses elicited by chemical stimulation of the rostral ventrolateral medulla in conscious, unrestrained rats. *Journal of the autonomic nervous system* **31**:185-190.

Bazil MK and Gordon FJ (1993) Sympathoexcitation from the rostral ventrolateral medulla is mediated by spinal NMDA receptors. *Brain research bulletin* **31**:273-278.

Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA (1990) Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proceedings of the National Academy of Sciences of the United States of America* **87**:1620-1624.

Benyo Z, Gille A, Bennett CL, Clausen BE and Offermanns S (2006) Nicotinic acid-induced flushing is mediated by activation of epidermal langerhans cells. *Molecular pharmacology* **70**:1844-1849.

Benyo Z, Gille A, Kero J, Csiky M, Suchankova MC, Nusing RM, Moers A, Pfeffer K and Offermanns S (2005) GPR109A (PUMA-G/HM74A) mediates nicotinic acid-induced flushing. *The Journal of clinical investigation* **115**:3634-3640.

Berridge MJ (1998) Neuronal calcium signaling. *Neuron* **21**:13-26.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Bindokas VP, Jordan J, Lee CC and Miller RJ (1996) Superoxide production in rat hippocampal neurons: selective imaging with hydroethidine. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **16**:1324-1336.

Brown BG, Zhao XQ, Chait A, Fisher LD, Cheung MC, Morse JS, Dowdy AA, Marino EK, Bolson EL, Alaupovic P, Frohlich J and Albers JJ (2001) Simvastatin and niacin, antioxidant vitamins, or the combination for the prevention of coronary disease. *The New England journal of medicine* **345**:1583-1592.

Canner PL, Berge KG, Wenger NK, Stamler J, Friedman L, Prineas RJ and Friedewald W (1986) Fifteen year mortality in Coronary Drug Project patients: long-term benefit with niacin. *Journal of the American College of Cardiology* **8**:1245-1255.

Carlson LA (2005) Nicotinic acid: the broad-spectrum lipid drug. A 50th anniversary review. *Journal of internal medicine* **258**:94-114.

Chapp AD, Gui L, Huber MJ, Liu J, Larson RA, Zhu J, Carter JR and Chen QH (2014) Sympathoexcitation and pressor responses induced by ethanol in the central nucleus of amygdala involves activation of NMDA receptors in rats. *American journal of physiology Heart and circulatory physiology* **307**:H701-709.

Ciriello J, Caverson MM and Polosa C (1986) Function of the ventrolateral medulla in the control of the circulation. *Brain research* **396**:359-391.

Cortese-Krott MM, Rodriguez-Mateos A, Kuhnle GG, Brown G, Feelisch M and Kelm M (2012) A multilevel analytical approach for detection and visualization of intracellular NO production and nitrosation events using diaminofluoresceins. *Free radical biology & medicine* **53**:2146-2158.

Dawson VL, Dawson TM, Bartley DA, Uhl GR and Snyder SH (1993) Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **13**:2651-2661.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

DePuy SD, Stornetta RL, Bochorishvili G, Deisseroth K, Witten I, Coates M and Guyenet PG (2013) Glutamatergic neurotransmission between the C1 neurons and the parasympathetic preganglionic neurons of the dorsal motor nucleus of the vagus. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **33**:1486-1497.

Dingledine R, Borges K, Bowie D and Traynelis SF (1999) The glutamate receptor ion channels. *Pharmacological reviews* **51**:7-61.

Gabor A and Leenen FH (2012) Central neuromodulatory pathways regulating sympathetic activity in hypertension. *J Appl Physiol (1985)* **113**:1294-1303.

Gaidarov I, Chen X, Anthony T, Maciejewski-Lenoir D, Liaw C and Unett DJ (2013) Differential tissue and ligand-dependent signaling of GPR109A receptor: implications for anti-atherosclerotic therapeutic potential. *Cellular signalling* **25**:2003-2016.

Garthwaite J, Charles SL and Chess-Williams R (1988) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. *Nature* **336**:385-388.

Greene LA (1978) Nerve growth factor prevents the death and stimulates the neuronal differentiation of clonal PC12 pheochromocytoma cells in serum-free medium. *The Journal of cell biology* **78**:747-755.

Guyenet PG (2006) The sympathetic control of blood pressure. *Nature reviews Neuroscience* **7**:335-346.

Hanson J, Gille A, Zwykiel S, Lukasova M, Clausen BE, Ahmed K, Tunaru S, Wirth A and Offermanns S (2010) Nicotinic acid- and monomethyl fumarate-induced flushing involves GPR109A expressed by keratinocytes and COX-2-dependent prostanoid formation in mice. *The Journal of clinical investigation* **120**:2910-2919.

Huang PH, Lin CP, Wang CH, Chiang CH, Tsai HY, Chen JS, Lin FY, Leu HB, Wu TC, Chen JW and Lin SJ (2012) Niacin improves ischemia-induced neovascularization in diabetic

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

mice by enhancement of endothelial progenitor cell functions independent of changes in plasma lipids. *Angiogenesis* **15**:377-389.

Ibrahim BM and Abdel-Rahman AA (2011) Role of brainstem GABAergic signaling in central cannabinoid receptor evoked sympathoexcitation and pressor responses in conscious rats. *Brain research* **1414**:1-9.

Ibrahim BM and Abdel-Rahman AA (2012) Enhancement of rostral ventrolateral medulla neuronal nitric-oxide synthase-nitric-oxide signaling mediates the central cannabinoid receptor 1-evoked pressor response in conscious rats. *The Journal of pharmacology and experimental therapeutics* **341**:579-586.

Ibrahim BM and Abdel-Rahman AA (2015) A pivotal role for enhanced brainstem Orexin receptor 1 signaling in the central cannabinoid receptor 1-mediated pressor response in conscious rats. *Brain research* **1622**:51-63.

Ito S, Hiratsuka M, Komatsu K, Tsukamoto K, Kanmatsuse K and Sved AF (2003) Ventrolateral medulla AT1 receptors support arterial pressure in Dahl salt-sensitive rats. *Hypertension* **41**:744-750.

Iwata J, Chida K and LeDoux JE (1987) Cardiovascular responses elicited by stimulation of neurons in the central amygdaloid nucleus in awake but not anesthetized rats resemble conditioned emotional responses. *Brain research* **418**:183-188.

Kagiyama S, Tsuchihashi T, Abe I and Fujishima M (1997) Cardiovascular effects of nitric oxide in the rostral ventrolateral medulla of rats. *Brain research* **757**:155-158.

Kish PE and Ueda T (1991) Calcium-dependent release of accumulated glutamate from synaptic vesicles within permeabilized nerve terminals. *Neuroscience letters* **122**:179-182.

Kubo T, Amano M and Asari T (1993) N-methyl-D-aspartate receptors but not non-N-methyl-D-aspartate receptors mediate hypertension induced by carotid body chemoreceptor

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

stimulation in the rostral ventrolateral medulla of the rat. *Neuroscience letters* **164**:113-116.

Kumagai H, Oshima N, Matsuura T, Iigaya K, Imai M, Onimaru H, Sakata K, Osaka M, Onami T, Takimoto C, Kamayachi T, Itoh H and Saruta T (2012) Importance of rostral ventrolateral medulla neurons in determining efferent sympathetic nerve activity and blood pressure. *Hypertension research : official journal of the Japanese Society of Hypertension* **35**:132-141.

Lin JC, Tsao WL and Wang Y (1995) Cardiovascular effects of NMDA in the RVLM of spontaneously hypertensive rats. *Brain research bulletin* **37**:289-294.

Lin LL, Lin AY and Knopf JL (1992) Cytosolic phospholipase A2 is coupled to hormonally regulated release of arachidonic acid. *Proceedings of the National Academy of Sciences of the United States of America* **89**:6147-6151.

Lipton SA and Rosenberg PA (1994) Excitatory amino acids as a final common pathway for neurologic disorders. *The New England journal of medicine* **330**:613-622.

Maciejewski-Lenoir D, Richman JG, Hakak Y, Gaidarov I, Behan DP and Connolly DT (2006) Langerhans cells release prostaglandin D2 in response to nicotinic acid. *The Journal of investigative dermatology* **126**:2637-2646.

Mao L and Abdel-Rahman AA (1994) Inhibition of glutamate uptake in the rostral ventrolateral medulla enhances baroreflex-mediated bradycardia in conscious rats. *Brain research* **654**:343-348.

Martins-Pinge MC, Araujo GC and Lopes OU (1999) Nitric oxide-dependent guanylyl cyclase participates in the glutamatergic neurotransmission within the rostral ventrolateral medulla of awake rats. *Hypertension* **34**:748-751.

Martins-Pinge MC, Garcia MR, Zoccal DB, Crestani CC and Pinge-Filho P (2007) Differential influence of iNOS and nNOS inhibitors on rostral ventrolateral medullary mediated

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

cardiovascular control in conscious rats. *Autonomic neuroscience : basic & clinical* **131**:65-69.

Medina-Ceja L, Pardo-Pena K, Morales-Villagran A, Ortega-Ibarra J and Lopez-Perez S (2015) Increase in the extracellular glutamate level during seizures and electrical stimulation determined using a high temporal resolution technique. *BMC neuroscience* **16**:11.

Montague PR, Gancayco CD, Winn MJ, Marchase RB and Friedlander MJ (1994) Role of NO production in NMDA receptor-mediated neurotransmitter release in cerebral cortex. *Science* **263**:973-977.

Montiel T, Camacho A, Estrada-Sanchez AM and Massieu L (2005) Differential effects of the substrate inhibitor l-trans-pyrrolidine-2,4-dicarboxylate (PDC) and the non-substrate inhibitor DL-threo-beta-benzyloxyaspartate (DL-TBOA) of glutamate transporters on neuronal damage and extracellular amino acid levels in rat brain in vivo. *Neuroscience* **133**:667-678.

Morrow JD, Parsons WG, 3rd and Roberts LJ, 2nd (1989) Release of markedly increased quantities of prostaglandin D2 in vivo in humans following the administration of nicotinic acid. *Prostaglandins* **38**:263-274.

Nishihara M, Hirooka Y, Matsukawa R, Kishi T and Sunagawa K (2012) Oxidative stress in the rostral ventrolateral medulla modulates excitatory and inhibitory inputs in spontaneously hypertensive rats. *Journal of hypertension* **30**:97-106.

Paxinos G, Watson CR and Emson PC (1980) AChE-stained horizontal sections of the rat brain in stereotaxic coordinates. *Journal of neuroscience methods* **3**:129-149.

Penberthy WT (2009) Nicotinic acid-mediated activation of both membrane and nuclear receptors towards therapeutic glucocorticoid mimetics for treating multiple sclerosis. *PPAR research* **2009**:853707.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Penugonda S, Mare S, Goldstein G, Banks WA and Ercal N (2005) Effects of N-acetylcysteine amide (NACA), a novel thiol antioxidant against glutamate-induced cytotoxicity in neuronal cell line PC12. *Brain research* **1056**:132-138.

Penumarti A and Abdel-Rahman AA (2014) The novel endocannabinoid receptor GPR18 is expressed in the rostral ventrolateral medulla and exerts tonic restraining influence on blood pressure. *The Journal of pharmacology and experimental therapeutics* **349**:29-38.

Pepponi R, Ferrante A, Ferretti R, Martire A and Popoli P (2009) Region-specific neuroprotective effect of ZM 241385 towards glutamate uptake inhibition in cultured neurons. *European journal of pharmacology* **617**:28-32.

Reigada D, Lu W and Mitchell CH (2006) Glutamate acts at NMDA receptors on fresh bovine and on cultured human retinal pigment epithelial cells to trigger release of ATP. *The Journal of physiology* **575**:707-720.

Robinson KM, Janes MS, Pehar M, Monette JS, Ross MF, Hagen TM, Murphy MP and Beckman JS (2006) Selective fluorescent imaging of superoxide in vivo using ethidium-based probes. *Proceedings of the National Academy of Sciences of the United States of America* **103**:15038-15043.

Separovic D, Kester M, Haxhiu MA and Ernsberger P (1997) Activation of phosphatidylcholine-selective phospholipase C by I1-imidazoline receptors in PC12 cells and rostral ventrolateral medulla. *Brain research* **749**:335-339.

Soga T, Kamohara M, Takasaki J, Matsumoto S, Saito T, Ohishi T, Hiyama H, Matsuo A, Matsushime H and Furuichi K (2003) Molecular identification of nicotinic acid receptor. *Biochemical and biophysical research communications* **303**:364-369.

Soudijn W, van Wijngaarden I and Ijzerman AP (2007) Nicotinic acid receptor subtypes and their ligands. *Medicinal research reviews* **27**:417-433.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Southam E, East SJ and Garthwaite J (1991) Excitatory amino acid receptors coupled to the nitric oxide/cyclic GMP pathway in rat cerebellum during development. *Journal of neurochemistry* **56**:2072-2081.

Spector R (1979) Niacin and niacinamide transport in the central nervous system. In vivo studies. *Journal of neurochemistry* **33**:895-904.

Sudhof TC (2004) The synaptic vesicle cycle. *Annual review of neuroscience* **27**:509-547.

Taggart AK, Kero J, Gan X, Cai TQ, Cheng K, Ippolito M, Ren N, Kaplan R, Wu K, Wu TJ, Jin L, Liaw C, Chen R, Richman J, Connolly D, Offermanns S, Wright SD and Waters MG (2005) (D)-beta-Hydroxybutyrate inhibits adipocyte lipolysis via the nicotinic acid receptor PUMA-G. *The Journal of biological chemistry* **280**:26649-26652.

Tingley FD, 3rd and Arneric SP (1990) Evidence for clonidine presynaptically modulating amino acid release in the rostral ventral medulla: role in hypertension. *Brain research* **537**:175-181.

Tunaru S, Kero J, Schaub A, Wufka C, Blaukat A, Pfeffer K and Offermanns S (2003) PUMA-G and HM74 are receptors for nicotinic acid and mediate its anti-lipolytic effect. *Nature medicine* **9**:352-355.

Vanhorn J, Altenburg JD, Harvey KA, Xu Z, Kovacs RJ and Siddiqui RA (2012) Attenuation of niacin-induced prostaglandin D(2) generation by omega-3 fatty acids in THP-1 macrophages and Langerhans dendritic cells. *Journal of inflammation research* **5**:37-50.

Wang WZ, Wang LG, Gao L and Wang W (2007) Contribution of AMPA/kainate receptors in the rostral ventrolateral medulla to the hypotensive and sympathoinhibitory effects of clonidine. *American journal of physiology Regulatory, integrative and comparative physiology* **293**:R1232-1238.

Wang Z, Wei X, Liu K, Zhang X, Yang F, Zhang H, He Y, Zhu T, Li F, Shi W, Zhang Y, Xu H, Liu J and Yi F (2013) NOX2 deficiency ameliorates cerebral injury through reduction of

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

complexin II-mediated glutamate excitotoxicity in experimental stroke. *Free radical biology & medicine* **65**:942-951.

Whitney EJ, Krasuski RA, Personius BE, Michalek JE, Maranian AM, Kolasa MW, Monick E, Brown BG and Gotto AM, Jr. (2005) A randomized trial of a strategy for increasing high-density lipoprotein cholesterol levels: effects on progression of coronary heart disease and clinical events. *Annals of internal medicine* **142**:95-104.

Wise A, Foord SM, Fraser NJ, Barnes AA, Elshourbagy N, Eilert M, Ignar DM, Murdock PR, Steplewski K, Green A, Brown AJ, Dowell SJ, Szekeres PG, Hassall DG, Marshall FH, Wilson S and Pike NB (2003) Molecular identification of high and low affinity receptors for nicotinic acid. *The Journal of biological chemistry* **278**:9869-9874.

Wu KL, Chao YM, Tsay SJ, Chen CH, Chan SH, Dovinova I and Chan JY (2014) Role of nitric oxide synthase uncoupling at rostral ventrolateral medulla in redox-sensitive hypertension associated with metabolic syndrome. *Hypertension* **64**:815-824.

Yang EJ, Kim GS, Jun M and Song KS (2014) Kaempferol attenuates the glutamate-induced oxidative stress in mouse-derived hippocampal neuronal HT22 cells. *Food & function* **7**:1395-1402.

Zanzinger J (2002) Mechanisms of action of nitric oxide in the brain stem: role of oxidative stress. *Autonomic neuroscience : basic & clinical* **98**:24-27.

Zhang J and Abdel-Rahman AA (2002) The hypotensive action of rilmenidine is dependent on functional N-methyl-D-aspartate receptor in the rostral ventrolateral medulla of conscious spontaneously hypertensive rats. *The Journal of pharmacology and experimental therapeutics* **303**:204-210.

Zhang J, El-Mas MM and Abdel-Rahman AA (2001) Imidazoline I(1) receptor-induced activation of phosphatidylcholine-specific phospholipase C elicits mitogen-activated protein kinase phosphorylation in PC12 cells. *European journal of pharmacology* **415**:117-125.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Footnotes

This research was supported by the Department of Pharmacology and Toxicology at the Brody School of Medicine, East Carolina University; and in part by the Zagazig Faculty of Pharmacy via a scholarship provided by the Egyptian Government (Scholarship Missions Program, Ministry of Higher Education); and the National Institutes of Health National Institute on Alcohol Abuse and Alcoholism [Grant 2R01-AA01444-09].

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Legends for Figures

Fig. 1. (A) Expression of GPR109A (42 kDa) in the rat RVLM or PC12 cells compared with expression in the spleen (positive control) and liver (negative control). Data are presented as integrated density ratio of GPR109A to the corresponding GAPDH values (*n* = 3) and expressed as mean ± S.E.M. **(B)** Dual-labeled IF of PC12 cells showing co-expression of GPR109A and TH.

Fig. 2. Confocal dual-labeled IF of post-fixed RVLM sections (*n* = 4-6) from naïve rats (*n* = 6) showing the spatial distribution of GPR109A in neurons, TH-ir neurons, astrocytes and microglia. Dual-channel images showing GPR109A (green) and one of the following markers (red): NeuN **(A)**, TH **(B)**, GFAP **(C)** or CD11b/c (**D**). In general, the 3 images for each section (A, B, C or D) show the same field at different magnifications where the two smaller images showing staining for different markers (e.g. A2 and A3) while the larger image (e.g. A1) shows the merged staining for these two markers. The arrows illustrate co-labeled cells. Scale bar, 20 μm. **(E)** quantitative analysis of A, B, C and D. Bar graph represents the total number of cells expressing different markers (open) and the number of cells co-labeled with GPR109A and each cell specific marker (filled) in the examined RVLM sections (mean ± S.E.M). CD11b/c: microglia marker; GFAP: astrocytes marker; NeuN: neurons marker; IF: immunofluorescence; RVLM: the rostral ventrolateral medulla; TH: tyrosine hydroxylase.

Fig. 3. Representative mean arterial pressure (MAP) and heart rate (HR) responses caused by intra-RVLM NA (20μg) or L-glutamate (169 ng) **(A)**. Effect of intra-RVLM NA (5, 10, or 20 *μ*g) on MAP **(B)** and HR **(C)** in conscious male rats. Values are mean change from base line ± S.E.M, (*n* = 4-7). **P* < 0.05 vs. ACSF. ACSF: artificial cerebrospinal fluid; ΔHR: change in HR; ΔMAP: change in mean arterial pressure; RVLM: the rostral ventrolateral medulla.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 4. Time-course changes in mean arterial pressure (ΔMAP) **(A)** and heart rate (ΔHR) **(B)** evoked by intra-RVLM NA or IsoNA (20 μg) compared with equal volume of ACSF in conscious male rats. **(C,D)**: time-course changes in ΔMAP and ΔHR evoked by intra-RVLM NA (20 μg) in conscious male rats pretreated, 10 min earlier, with ACSF, NMDA receptor blocker (AP5, 2 nmol/100nl) or L-glutamate uptake inhibitor (PDC, 1 μM). **(E)** The area under the curve (AUC) data generated from 20 min BP time-course values for different treatments. Data from AP5 (2 nmol/100nl) or PDC (1 μM) treated rats were not significantly different from the control values, and are not shown for clarity. Values are mean change from base line ± S.E.M (n = 5-7). *P < 0.05 vs. ACSF values; $^{\#}P$ < 0.05 vs. NA. ACSF: artificial cerebrospinal fluid; BP: blood pressure; IsoNA: iso-nicotinic acid; RVLM: the rostral ventrolateral medulla.

Fig. 5. (A) DCFH-DA measured ROS levels in terms of relative fluorescence units (RFU) of produced DCF in the RVLM after ACSF or NA (20 μg) injection. **(B)** Effect of ACSF, NA (20 μg), AP5 (2 nmol)/NA (20 μg), CNQx (200 pmol)/NA (20 μg), PDC (1 μM)/NA (20 μg) and isoNA (20 μg) on RVLM ROS levels detected by DHE staining (visualized with confocal microscopy and quantified using Zen Lite 2011 software). Values are mean ± S.E.M. (n = 4-5 rats). *P < 0.05 vs. ACSF values; $^{\#}P$ < 0.05 vs. NA. ACSF: artificial cerebrospinal fluid; IsoNA: iso-nicotinic acid; NA: nicotinic acid; RVLM: the rostral ventrolateral medulla.

Fig. 6. (A) Effect of NA (10 μM, 100 μM, and 1mM), or IsoNA (1mM) on Ca^{2+} levels in PC12 cells. Measurements were done 30 secs, and 2 and 4 min incubation time. Data representing Ca^{2+} concentration (μM) is expressed as mean ± S.E.M. **(B)** Effect of 1 min exposure of PC12 cells to NA or IsoNA (1mM) on L-glutamate levels (folds increase of control) with or without 1 h prior incubation with PDC (0.1 mM). **(C)** NO DAF-FM fluorescence intensity in PC12 cells incubated with NA (10 μM, 100 μM, 1mM) or IsoNA (1mM) for 1 min. Data is expressed as

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

mean ± S.E.M. *P < 0.05 vs. control values; $^{\#}P$ < 0.05 vs. PDC; $^{\wedge}P$ < 0.05 vs. NA. IsoNA: iso-nicotinic acid; NA: nicotinic acid; NO: nitric oxide.

Fig. 7. (A) Effect of NA (10 μM, 100 μM, and 1mM), or IsoNA (1mM) on DCFH-DA measured ROS generation in PC12 cells (folds increase from control). **(B)** Effect of NA (1mM) on DCFH-DA measured ROS generation compared to glutamate (100nM) with or without 1 h or 10 min prior incubation with PDC (0.1 mM), or AP5 (100 μM), respectively. Kinetic measurements were recorded for 30 min following different treatments. Data is expressed as mean ± S.E.M. *P < 0.05 vs. control. IsoNA: iso-nicotinic acid; L-glut.: L-glutamate; NA: nicotinic acid.

Fig. 8. (A) Western blot for GPR109A in siRNA transfected cells compared to control (n = 3). Data are presented as integrated density ratio of GPR109A to the corresponding β-actin values, and expressed as mean ± S.E.M. Effect of vehicle or NA (1mM) on Ca^{2+} **(B)** and glutamate **(C)** levels in PC12 cells pre-incubated for 24 h with either scrambled or GPR109A siRNA. Measurements were done at 30 sec and 1 min incubation times for the measurements of Ca^{2+} and glutamate, respectively, and represented as fold increase from corresponding control. Data is expressed as mean ± S.E.M. *P < 0.05 vs. control values. NA: nicotinic acid.

Fig. 9. Suggested mechanisms for the GPR109A-mediated pressor response caused by nicotinic acid (NA) microinjection into the RVLM. The sequence of events is based on the findings that: (i) Intra-RVLM GPR109A activation (NA) increased blood pressure in conscious freely moving rats (Fig. 3), and increased RVLM ROS levels (Fig. 5); (ii) NA increased Ca^{+2}, glutamate NO and ROS levels (Figs. 6 and 7) in PC12 cells, which exhibit a neuronal phenotype. The dashed arrows indicate proposed signaling (see text for details).

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

TABLE 1

Mean arterial pressure (MAP, mm Hg) and heart rate (HR, beats/min) values immediately before intra-RVLM treatment with the indicated intervention or the vehicle (ACSF).

Pretreatment/Treatment	Rats per group (n)	MAP (mmHg)	HR (beats/min)
ACSF	7	126.1 ± 5.501	341.7 ± 12.36
IsoNA	6	133.7 ± 4.219	336.3 ± 6.224
ACSF/Nicotinic acid	7	122.6 ± 5.086	353.7 ± 18.19
AP5/Nicotinic acid	5	127.2 ± 6.258	379.3 ± 26.75
PDC/Nicotinic acid	7	125.0 ± 3.070	326.7 ± 30.92
CNQX/Nicotinic acid	5	126.8 ± 4.737	340.2 ± 24.97

ACSF, artificial cerebrospinal fluid; IsoNA, iso-nicotinic acid; RVLM, rostral ventrolateral medulla. Values are the mean ± S.E.M.

Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

Fig. 1

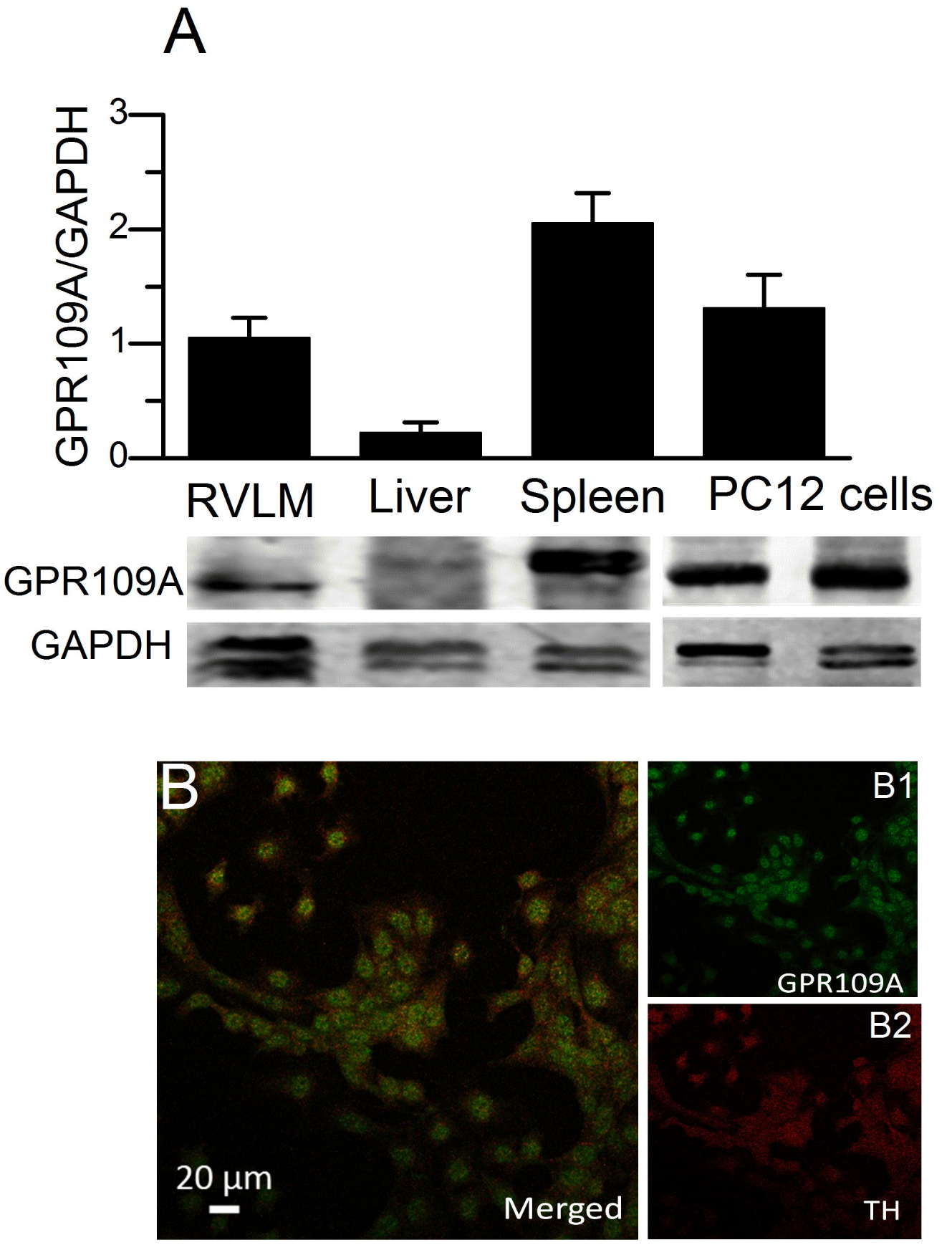
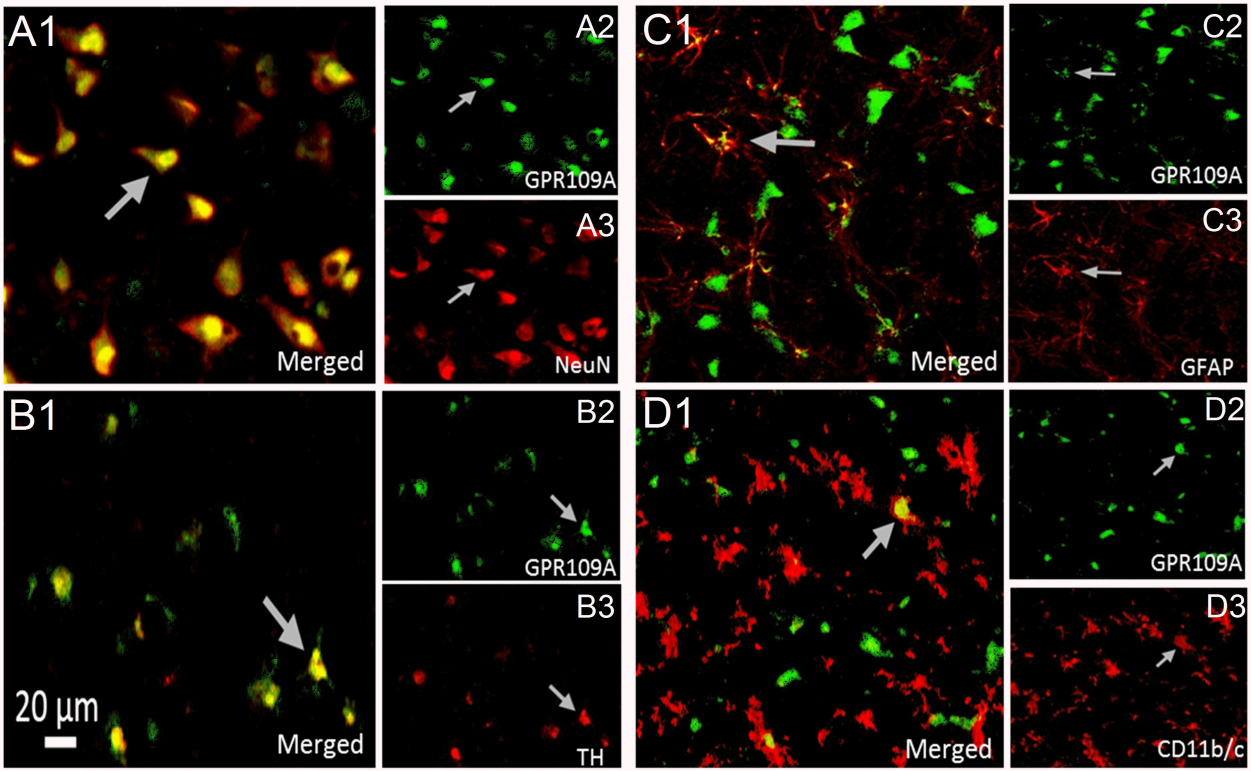

A
GPR109A/GAPDH
3
2
1
0
RVLM
Liver
Spleen
PC12 cells
GPR109A
GAPDH
B
20 μm
Merged
B1
GPR109A
B2
TH

Fig. 2


A1
Merged
A2
GPR109A
A3
NeuN
C1
Merged
C2
GPR109A
C3
GFAP
B1
20 µm
Merged
B2
GPR109A
B3
TH
D1
Merged
D2
GPR109A
D3
CD11b/c

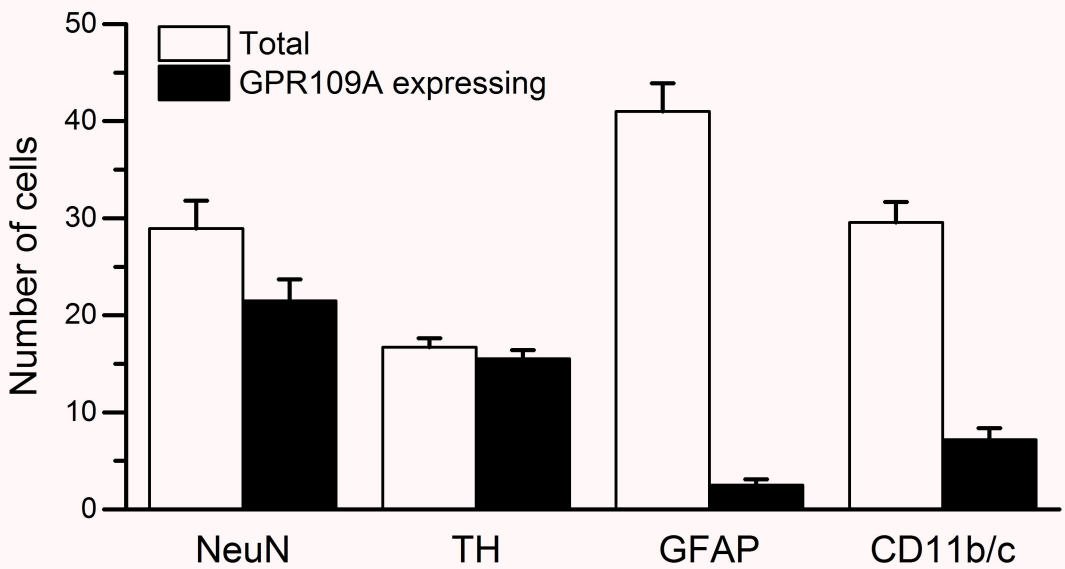

Total
GPR109A expressing
Number of cells
50
40
30
20
10
0
NeuN
TH
GFAP
CD11b/c

Fig. 3

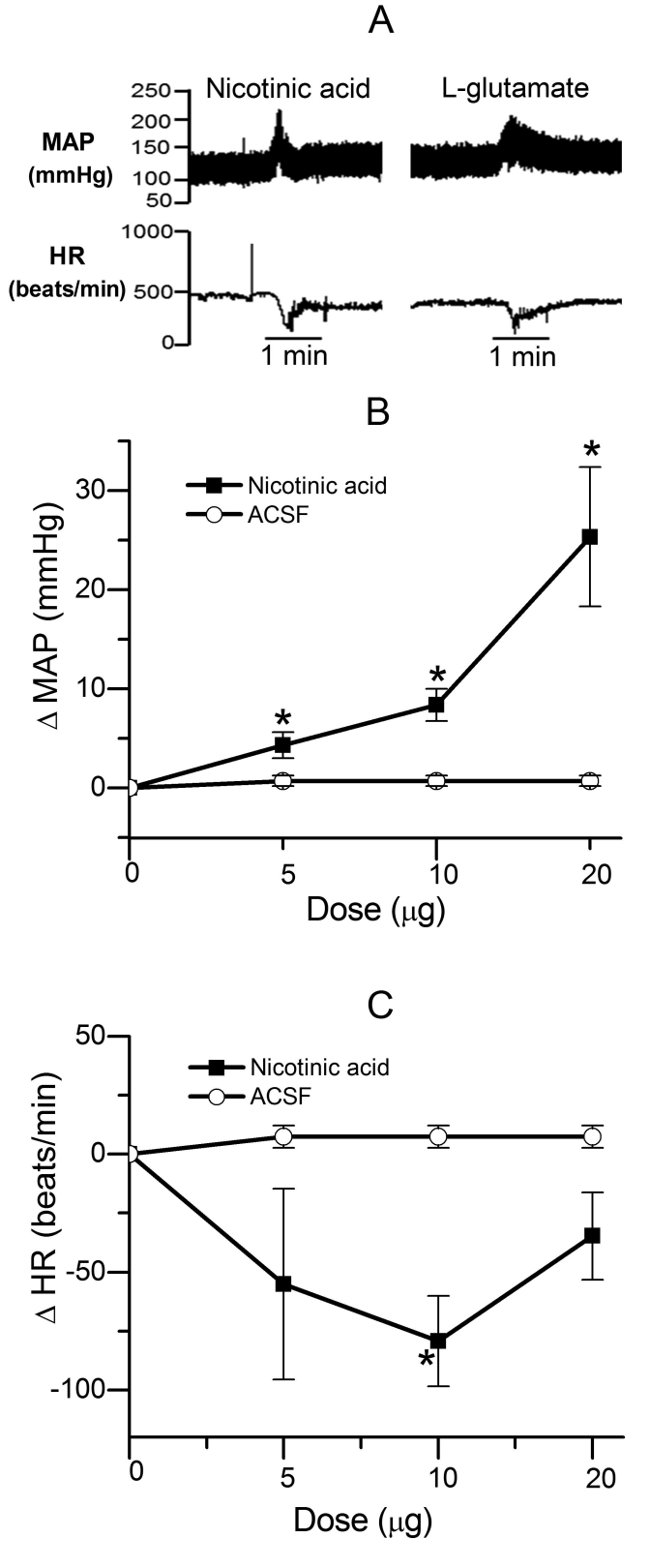

A
Nicotinic acid
L-glutamate
MAP
(mmHg)
250
200
150
100
50
HR
(beats/min)
1000
500
0
1 min
1 min
B
Nicotinic acid
ACSF
Δ MAP (mmHg)
30
20
10
0
*
*
*
0
5
10
20
Dose (μg)
C
Nicotinic acid
ACSF
Δ HR (beats/min)
50
0
-50
-100
*
0
5
10
20
Dose (μg)

Fig. 4

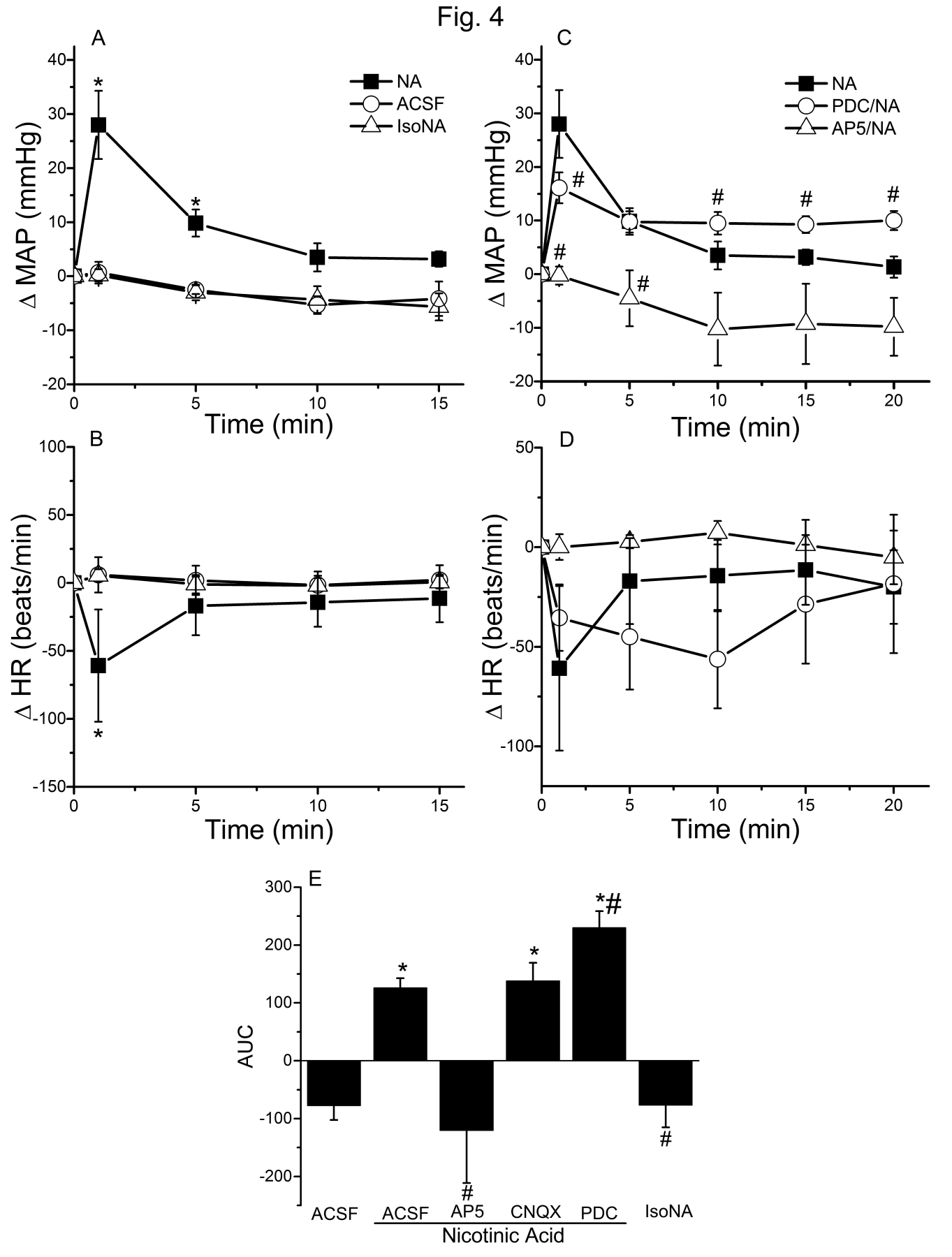
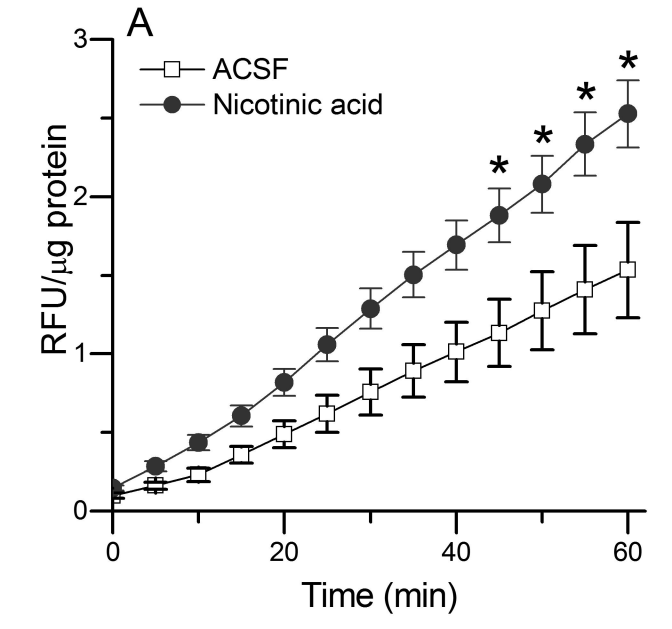

A
NA
ACSF
IsoNA
Δ MAP (mmHg)
Time (min)
B
Δ HR (beats/min)
Time (min)
C
NA
PDC/NA
AP5/NA
Δ MAP (mmHg)
Time (min)
D
Δ HR (beats/min)
Time (min)
E
AUC
ACSF
ACSF
AP5
CNQX
PDC
IsoNA
Nicotinic Acid

Fig. 5


A
ACSF
Nicotinic acid
RFU/μg protein
Time (min)
0
1
2
3
0
20
40
60
*

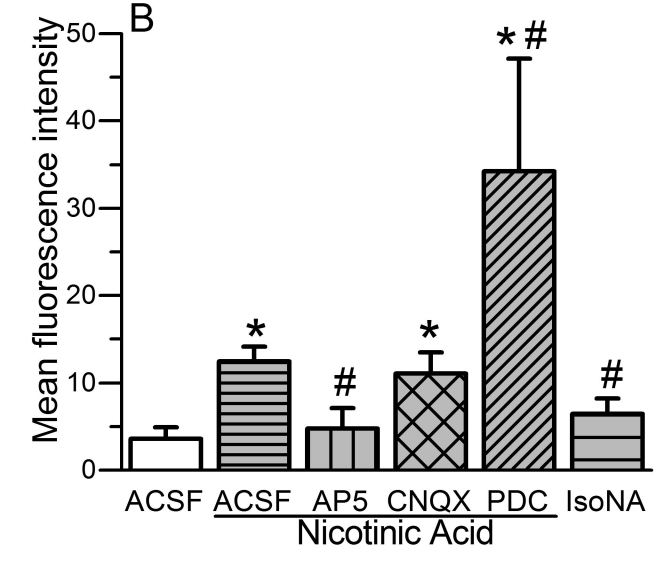
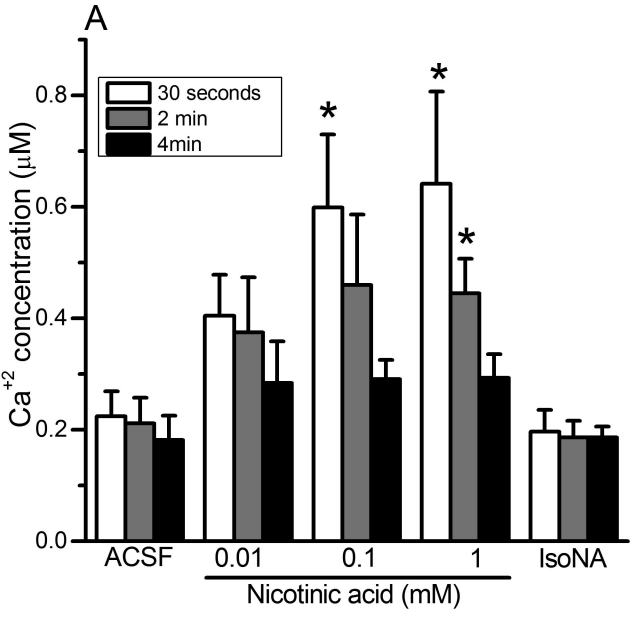
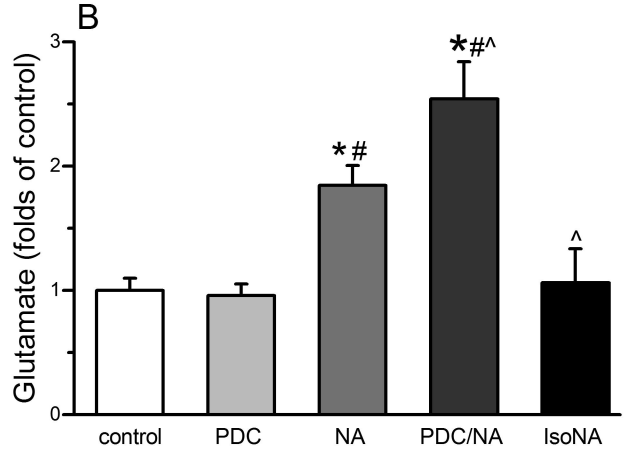

B
Mean fluorescence intensity
0
10
20
30
40
50
*
*
#
*#
#
ACSF
ACSF
AP5
CNQX
PDC
IsoNA
Nicotinic Acid

Fig. 6


A
30 seconds
2 min
4min
Ca+2 concentration (μM)
0.0
0.2
0.4
0.6
0.8
*
*
*
ACSF
0.01
0.1
1
IsoNA
Nicotinic acid (mM)


B
Glutamate (folds of control)
0
1
2
3
*#
*#^
^
control
PDC
NA
PDC/NA
IsoNA

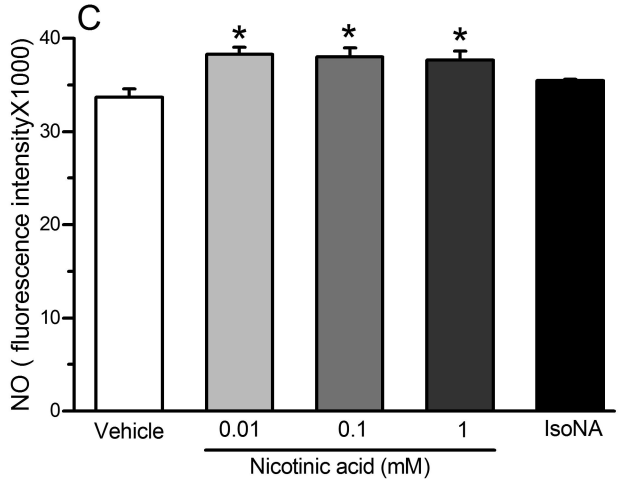
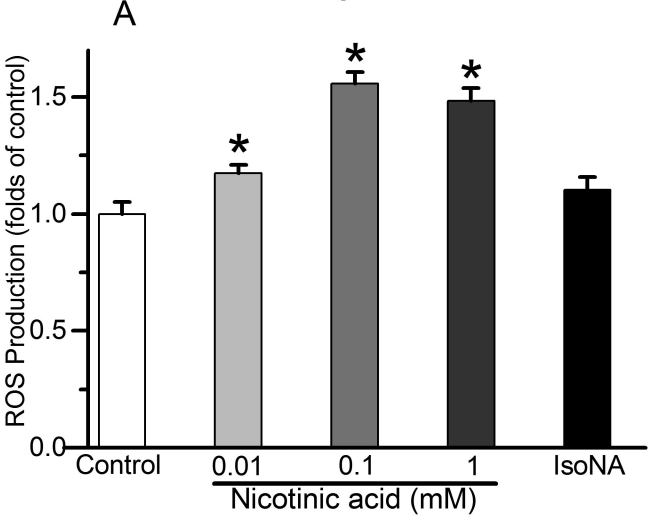
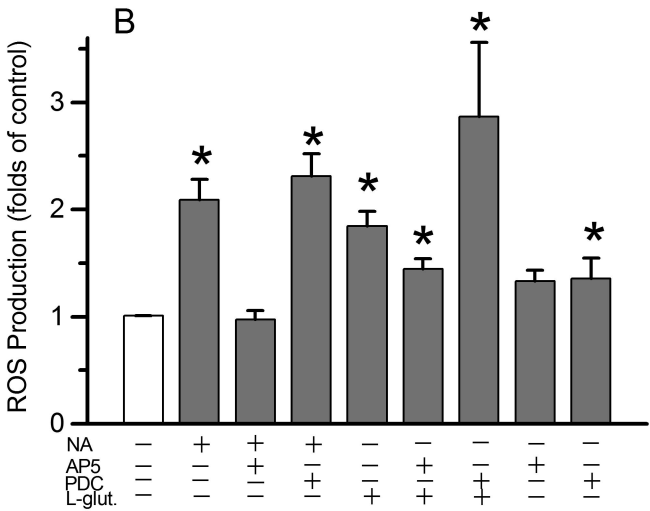

C
NO (fluorescence intensityX1000)
0
10
20
30
40
*
*
*
Vehicle
0.01
0.1
1
IsoNA
Nicotinic acid (mM)

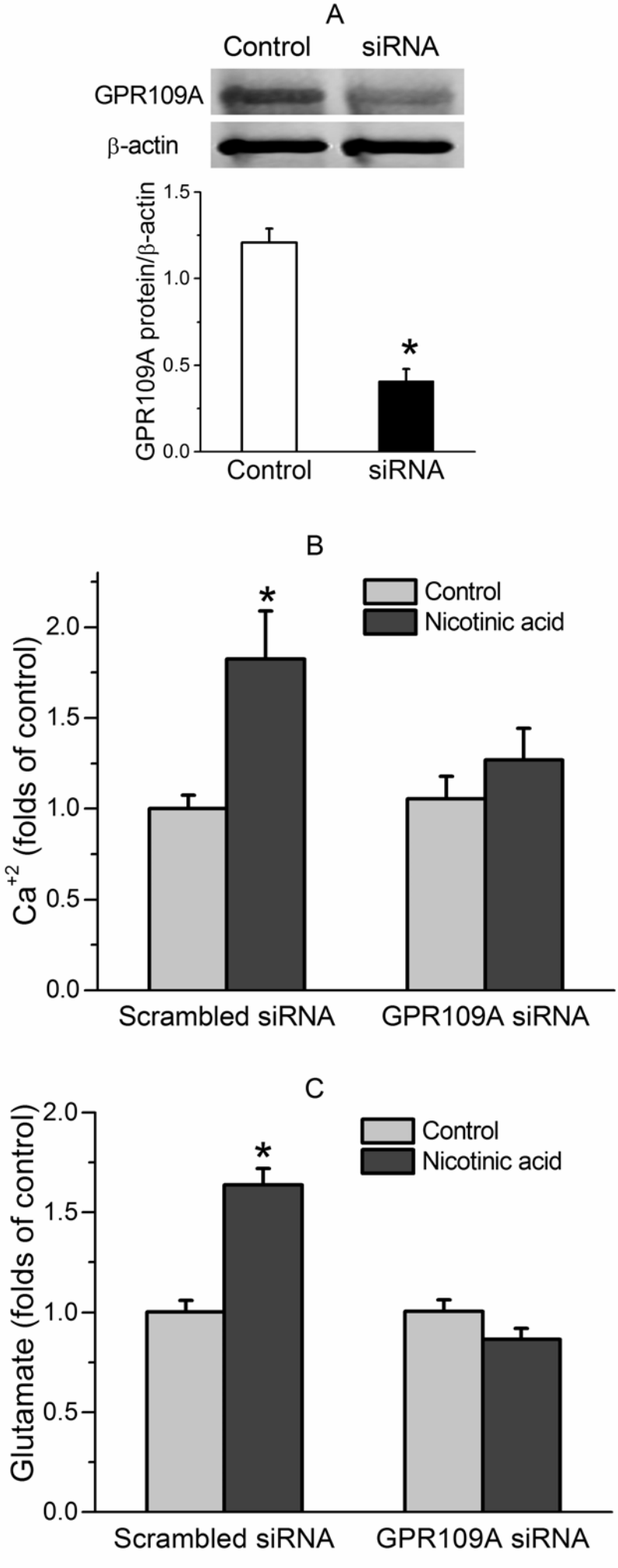
Fig. 7


A
ROS Production (folds of control)
1.5
1.0
0.5
0.0
Control
0.01
0.1
1
IsoNA
Nicotinic acid (mM)


B
ROS Production (folds of control)
3
2
1
0
NA
AP5
PDC
L-glut.

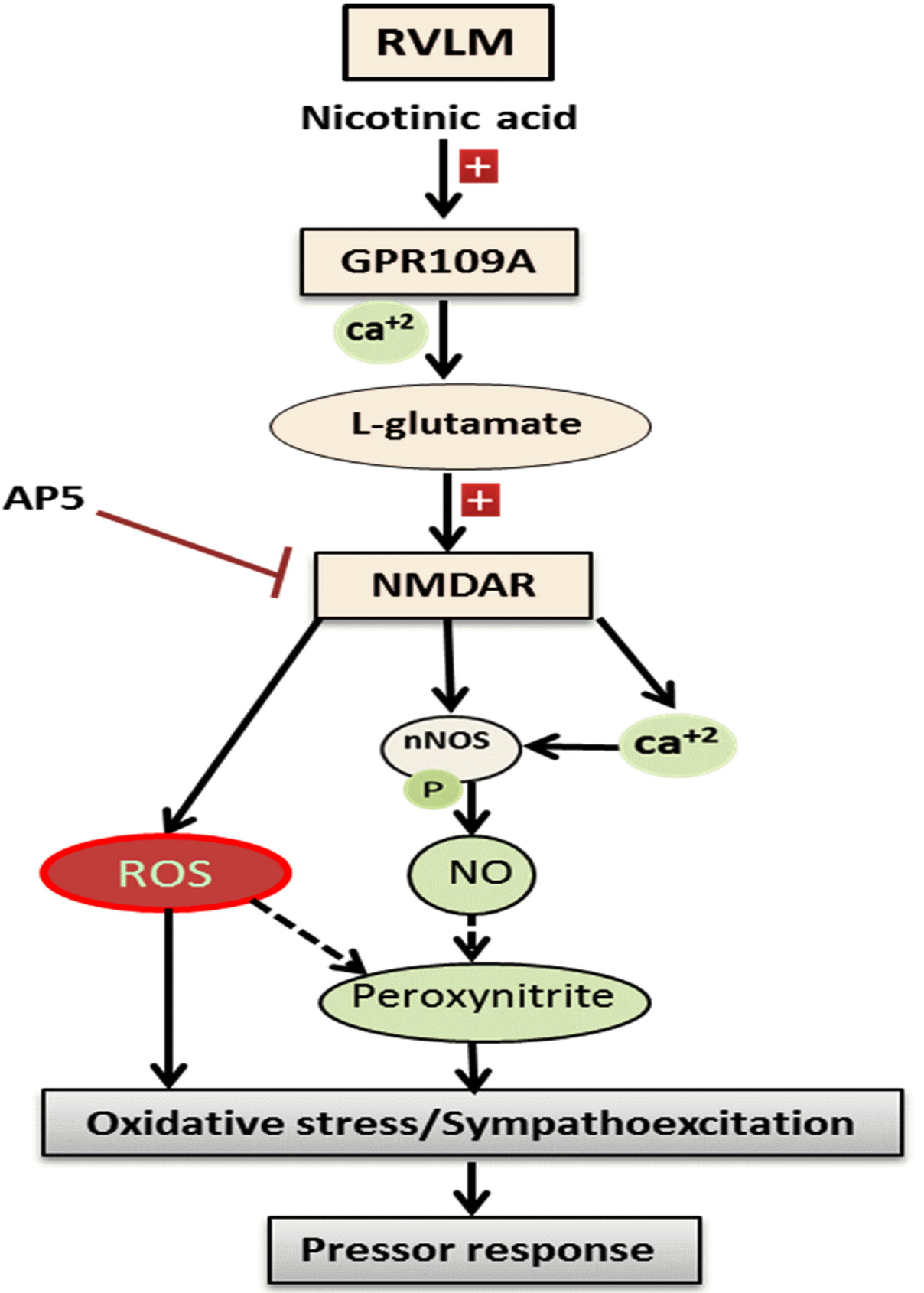
JPET Fast Forward. Published on November 30, 2015 as DOI: 10.1124/jpet.115.229146
This article has not been copyedited and formatted. The final version may differ from this version.

Fig. 8



Downloaded from jpet.aspetjournals.org at ASPET Journals on March 9, 2022

Fig. 9


RVLM
Nicotinic acid
+
GPR109A
ca+2
L-glutamate
AP5
+
NMDAR
nNOS
ca+2
P
ROS
NO
Peroxynitrite
Oxidative stress/Sympathoexcitation
Pressor response