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

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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).

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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.
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,
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.
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% CO₂. 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).
**Measurement of 
Ca^{2+}, Glutamate, and NO Measurements.**

(Ca^{2+}) 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
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
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
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.
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 ($P < 0.05$)
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 interventions 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
(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).
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 Ca2+, 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
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
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 sympatheoxcitation 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
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
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.
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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.
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Footnotes

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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 µg) 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.
**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
mean ± S.E.M. *P < 0.05 vs. control values; ¤P < 0.05 vs. PDC; ^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).
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).

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

ACSF, artificial cerebrospinal fluid; IsoNA, iso-nicotinic acid; RVLM, rostral ventrolateral medulla. Values are the mean ± S.E.M.
Fig. 1

A

![Bar chart and Western blot images showing the expression of GPR109A/GAPDH in different tissues: RVLM, Liver, Spleen, and PC12 cells.]

B

![Immunofluorescence images showing the localization of GPR109A and TH in a tissue section. The scale bar is 20 µm.]

**Merged**

GPR109A

TH
Fig. 2

(A1) Merged

(A2) GPR109A

(A3) NeuN

(C1) Merged

(C2) GPR109A

(C3) GFAP

(B1) Merged

(B2) GPR109A

(B3) TH

(D1) Merged

(D2) GPR109A

(D3) CD11b/c

20 μm

Graph:

- Total
- GPR109A expressing

<table>
<thead>
<tr>
<th></th>
<th>NeuN</th>
<th>TH</th>
<th>GFAP</th>
<th>CD11b/c</th>
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<tbody>
<tr>
<td>Number of cells</td>
<td></td>
<td></td>
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</table>
Fig. 8

A

Control  siRNA

GPR109A

β-actin

GPR109A protein/β-actin

Control  siRNA

B

Control  Nicotinic acid

Ca^{2+} (folds of control)

Scrambled siRNA  GPR109A siRNA

C

Control  Nicotinic acid

Glutamate (folds of control)

Scrambled siRNA  GPR109A siRNA
Fig. 9

RVLM

Nicotinic acid

GPR109A

$\text{ca}^{+2}$

L-glutamate

NMDAR

$\text{ca}^{+2}$

nNOS

ROS

NO

Peroxynitrite

Oxidative stress/Sympathoexcitation

Pressor response

AP5