34 Glutamatergic Receptor Activation in the Commisural Nucleus Tractus Solitarii (cNTS) Mediates Brain Glucose Retention (BGR) Response to Anoxic Carotid Chemoreceptor (CChr) Stimulation in Rats

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Abstract

Glutamate, released from central terminals of glossopharyngeal nerve, is a major excitatory neurotransmitter of commissural nucleus tractus solitarii (cNTS) afferent terminals, and brain derived neurotrophic factor (BDNF) has been shown to attenuate glutamatergic AMPA currents in NTS neurons. To test the hypothesis that AMPA contributes to glucose regulation *in vivo* modulating the hyperglycemic reflex with brain glucose retention (BGR), we microinjected AMPA and NBQX (AMPA antagonist) into the cNTS before carotid chemoreceptor stimulation in anesthetized normal Wistar rats, while hyperglycemic reflex an brain glucose retention (BGR) were analyzed. To investigate the underlying mechanisms, GluR2/3 receptor and c-Fos protein expressions in cNTS neurons were determined. We showed that AMPA in the cNTS before CChr stimulation inhibited BGR observed in aCSF group. In contrast, NBQX in similar conditions, did not

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modify the effects on glucose variables observed in aCSF control group. These experiments suggest that glutamatergic pathways, via AMPA receptors, in the cNTS may play a role in glucose homeostasis.

Keywords
AMPA • NBQX • Carotid body • Glucose homeostasis • Brain glucose retention

34.1 Introduction

There is strong evidence suggesting that AMPA receptors are involved in the central pathways to modulate chemoreceptor inputs, however the studies examining its role in the control of energy metabolism are scarce (Pang and Han 2012). It is known that excitatory synaptic transmission in the cardio-respiratory reflexes is mediated predominantly by the activation of AMPA receptors in the NTS to undertake a sympatho-excitation reflexes (Ozawa et al. 1998; Lin 2009). Ohtake studies (1998) suggest that AMPA ionotropic receptors are the main ventilatory response modulators. Furthermore, activation of AMPA receptor increases chemoreceptor responses evoked by stimulation in animals exposed to intermittent hypoxia (de Paula et al. 2007). In the same way, glutamate seems to be released from central terminals of glossopharyngeal afferents in the NTS [from the espinomedular junction to the caudal region of the facial motor nucleus in the rostral ventrolateral medulla (RVLM)], which plays a role in regulating sympathetic activity of baro- chemoreflex in respiratory function (Koshiya and Guyenet 1996). We addressed whether glutamate signaling, through AMPA receptors, modulates glucose homeostasis altered by anoxic stimulation of circulatorily isolated CChrs with cyanide (NaCN) (Alvarez-Buylla and Roces de Alvarez-Buylla 1994).

34.2 Methods

34.2.1 Animals and Surgical Procedures

Male Wistar rats (280–300 g) were used. All procedures were in accord to the Guide for the Care and Use of Laboratory Animals from National Institutes of Health, USA. Rats were anesthetized with a bolus injection of sodium pentobarbital (3 mg/100 g i.p.) supplemented by a continuous i.p. infusion of the same anesthetic (0.063 mg/min). Buprenorphine (0.03 mg/kg subcutaneously, Temgesic, Schering-Plough, México) 5 min before surgical procedures was used as analgesic. Body temperature was kept at 37 ± 1 °C with a heating pad. Animals were artificially ventilated, respiratory rate and tidal volume were based on pH, pO_2 and pCO_2 values in arterial blood obtained during experimental procedures, as well as 10 min before and at the end of experiment. Permanent silastic catheters filled with heparin (1,000 U/mL) were inserted into the abdominal aorta (accessed from the femoral artery) and jugular sinus (accessed from the right external jugular vein) without interrupting circulation in these vessels (Alvarez-Buylla and Alvarez-Buylla 1988). The correct placement of catheters was verified at the end of each experiment (Fig. 34.1a).

34.2.2 Drugs

The drugs used were: (a) sodium cyanide (NaCN); (Sigma, Mex.) at a dose of 5 μg/100 g (diluted in 100 nL of freshly prepared sterile saline-sal); (b) artificial cerebrospinal fluid (aCSF- 100 nL, containing NaCl 145 mM, KCl 2.7 mM, MgCl mM 1.0, $CaCl_2$ mM 1.2, ascorbate 2.0 mM, NaH_2PO_4 2, mM, pH 7.3–7.4); (c) α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA, 2 μM/100 nL of aCSF) (Sigma, Méx.) (Müeller et al. 2005); (c) or 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-[*f*]quinoxaline-7-sulfonamide, (NBQX, 2 mM/100 nL of aCSF), (Sigma, Méx.) (Müeller et al. 2005).

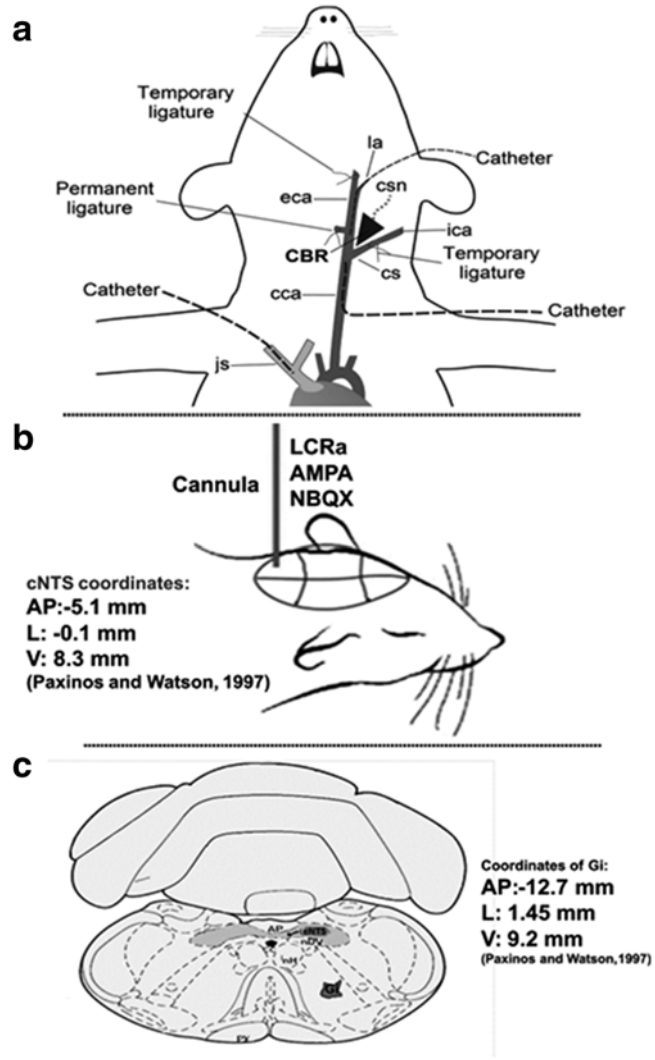

Fig. 34.1 (**a**) Placement of catheters and ligatures to locally perfuse left carotid sinus to stimulate carotid body chemoreceptors with NaCN. *cc* common carotid artery, *CBR* carotid body receptors, *cs* circulatory isolated carotid sinus, *csn* carotid sinus nerve,*eca* external carotid artery, *ica* internal carotid artery, *js* jugular sinus, *la* lingual artery. (**b**) Placement of cannula into the cNTS. (**c**) microinjection site stained with methylene blue (cNTS) (overdraw of Paxinos and Watson 1997)

Drugs were diluted immediately before application. In sham experiments, the same volume of aCSF was injected.

34.2.3 CChr Stimulation

CChr stimulation was performed as previously described (Alvarez-Buylla and Alvarez-Buyllla 1988). Briefly, 5 μg/100 g NaCN in 100 μL sal./2 s was injected into the local circulation of the left carotid sinus, avoiding baroreceptor stimulation. NaCN was used as a carotid body chemoreceptor stimulator since its effects are equivalent to those observed in anoxemic anoxia (Serani et al. 1983). The left carotid sinus was temporarily isolated from the cephalic circulation, while the right carotid sinus was denervated. With this technique only the left carotid sinus is exposed to NaCN and within 10–15 s, NaCN is cleared into a washing cannula (Fig. 34.1a). Previous experiments conducted on isolated chemoreceptor fibers showed that an injection of 5 μg/100 g of NaCN into the carotid body circulation elicits electrical activity before the first signs of respiratory changes are observed. NaCN administered as described above, but after carotid nerve section did not have any effect (Alvarez-Buylla and Alvarez-Buylla 1988).

34.2.4 Microinjection of Drugs into the cNTS

Injections (aCSF, AMPA or NBQX) into the cNTS were done using a stereotaxic frame (Stoelting Co., Wood Dale, Il, USA) and the following coordinates from bregma: AP = −5.1 mm, L = −0.1 mm, V = 8.3 mm; incisor bar 3.3 mm above zero point (Paxinos and Watson 1997). The surface of the brain was reached with a 1/32 in. burr hole and a glass micropipette (50–60 µm external tip diameter); filled with the solution to be injected, was inserted into the left cNTS (Fig. 34.1b). The micropipette was connected to a 0.5 mL Hamilton microsyringe with polyethylene tubing (PE 20) for injections. AMPA and NBQX drugs were delivered in 100 nL of artificial cerebrospinal fluid (aCSF) during 5 s approximately. The volume of each injection was determined by measuring the movement of the fluid meniscus within the microinjector pipette. In control experiments the micropipette was directed to the gigantocellular reticular nucleus (Gi), the coordinates in this case were AP = −12.7 mm, L = 1.45 mm, V = 9.2 mm (Paxinos and Watson 1997). Once the last blood sample was drawn, the correct positioning of the micropipette tip site was corroborated by injecting 50–100 nL of methylene blue (10 %) through the same micropipette. Anesthetized rats were decapitated; the brains were removed, immediately frozen, and sectioned at 40 µm in a cryostat (CM-1800, Leica Microsystems, Nussloch, Germany). Sections were stained with cresyl violet for histological verification of the microinjection site and tissue damage (Fig. 34.1c).

34.2.5 Blood Sampling and Measurements

Blood samples were taken via catheters inserted into the abdominal aorta (arterial) and from the jugular sinus (venous). Blood samples were collected from each rat as follows: two basal samples at t = −10 min and t = −5 min (to obtain a basal level in t = −7.5 min). AMPA, and NBQX drugs or aCSF, as control, were injected into the NTS at t = −4 min, while CChr stimulation was done into the local circulation of the isolated carotid sinus at t = 0 min. Four experimental samples were then collected at 5, 10, 20 and 30 min. To compensate for fluid loss, rats received 0.3 mL sal. after each sample was taken (0.15 mL of arterial blood and 0.15 mL of venous blood). Blood was centrifuged and plasma was kept chilled until assayed. Plasma glucose concentration in µmol/mL was determined by the glucose-oxidase method with a glucose analyzer (Beckman Autoanalyzer, Beckman Coulter, CA USA). Brain glucose retention was determined by arterial-venous (A-V) glucose differences between abdominal aorta and jugular sinus blood in µmol/mL. Blood flow was not considered for glucose retention because it does not change significantly after CChr stimulation (Alvarez-Buylla et al. 1997). To discard any possible change in blood pressure after NaCN injection into the carotid sinus circulation, arterial blood pressure was measured in the femoral artery in two control experiments with a pressure transducer (Ohmeda Pte Ltd, Singapore), the results in these experiments did not show significant changes; Glucose, PO_2, PCO_2 and pH values were within the range of standard curves at all times.

34.2.6 Experimental Protocol

Animals were allowed to stabilize for 30 min after surgery, at which time they were randomized into the following groups: (a) aCSF (100 nL into the cNTS) followed by CChr, n = 8; (b) AMPA (2 µM diluted in 100 nL aCSF into de cNTS) followed by CChr stimulation, n = 9; (c) NBQX (2 mM diluted in 100 nL aCSF into the NTS) followed by CChr stimulation, n = 9 (Fig. 34.2).

34.2.7 Immunohistochemistry for GluR2/3 and c-Fos

In aCSF, AMPA, and NBQX groups vibratome sections were processed for immunohistochemical staining. Collected sections were

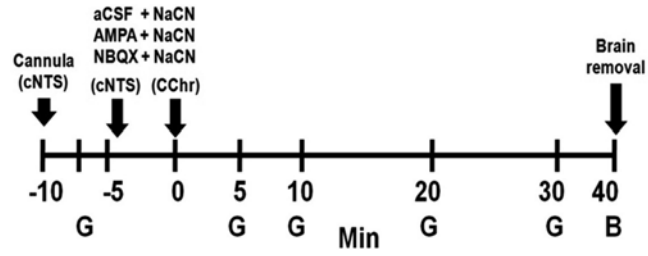

Fig. 34.2 Time line in experimental protocol. G, glycemia

soaked in phosphate-buffered saline (PBS; pH 7.3) for 12–24 h, washed in 3 % goat serum and 0.4 % triton X (GS-T) in PBS (3 % GS-T-PBS) for 1 h, and then incubated for 16–36 h at 4 °C in rabbit anti-GluR2/3 (1:100 dilution, Chemicon, Temecula, CA) and anti-c-Fos primary antibodies (1:1,000 dilution, Santa Cruz Biotechnology, CA), diluted in 3 % GS-T-PBS. Sections rinsed extensively in 3 % GST-PBS, and incubated (in both cases) in goat anti-rabbit biotinylated IgG secondary antibody (1:250, diluted in 3 % GS-T-PBS) (Jackson Immunoresearch, Westgrove, PA) for 2 h. The tissue was placed for 1.5 h at room temperature in avidin–biotinylated- peroxidase complex (1 % GS-T-PBS, ABC Vectastin Kit) (Vector Labs., Burlingame, CA), rinsed in PBS 0.1 M without triton. The peroxidase reaction product was visualized following a 10 min incubation in a chromogen solution (3.3 diaminobencidine 0.07 % and H_2O_2 0.01 %, pH 7.6) (Aldrich, St. Louis, MO) to obtain a brown color. After successive washes in PBS 0.1 M, sections were transferred to 0.05 M PBS, mounted, dehydrated, and coverslipped (Vectamount). Control sections (without the primary antibody) were processed as described. Tissue sections from each treatment group were pooled, and processed in tandem with control groups, to minimize variations in immunohistochemistry labeling. Sections were analyzed under an Axio Imager bright-field microscope equipped with a digital camera using AxioVision (version 4.8, 2009) software (Carl Zeiss, Munich, Germany). For each rat, 20 representative digital photomicrographs were taken at the same A/P coordinates using a Plan Achromat 20× objective. Positive GluR2/3 and Fos-ir cells were counted semiautomatically with a program using the Auto Measure Program Wizard (AxioVision version 4.8).

34.2.8 Data Analysis

Values expressed as means ± SEM. Data were analyzed using the SPSS 12.0 and ANOVA one way for multiple comparisons, and Scheffé's test to compare the data between groups. Significance was set at $p < 0.05$. Arterial glucose or BGR levels vs. their basal values (Student *t*-test), represented by an asterisk (*);plus sign (+) compares aCSF group vs. corresponding AMPA or NBQX groups. Comparison between AMPA and NBQX groups is represented by the pound sign (#). The actual basal arterial blood glucose values were 6.16 ± 0.16 µmol/mL (aCSF group), 6.0 ± 0.19 (AMPA group) and 5.88 ± 0.24 (NBQX group).

34.3 Results

When aCSF (100 nL) was injected into the cNTS 4 min before CChr stimulation with NaCN, an increase in arterial glucose concentration was observed at all the times studied, as previously shown (Alvarez-Buylla and Alvarez-Buylla 1988), the values rose from 6.33 ± 0.20 up to 9.88 ± 0.45 µmol/mL a t = 30 min ($p < 0.01$) (Fig. 34.3). When BGR was calculated, a prompt and significant increase was also obtained, with maximum level at t = 20 min, the values rose from 0.85 ± 0.08 µmol/mL up to 2.36 ± 0.23 µmol/mL ($p < 0.01$) (Fig. 34.4a). Rats that received an AMPA injection into the cNTS before CChr stimulation decreased their glucose levels when compared to aCSF group, with the higher decrement value at t = 5 min ($p < 0.01$), the values decreased from 6.01 ± 0.14 to 6.11 µmol/mL a t = 5 min ($p < 0.01$) (Fig. 34.4a). Similar results were observed in relation to BGR

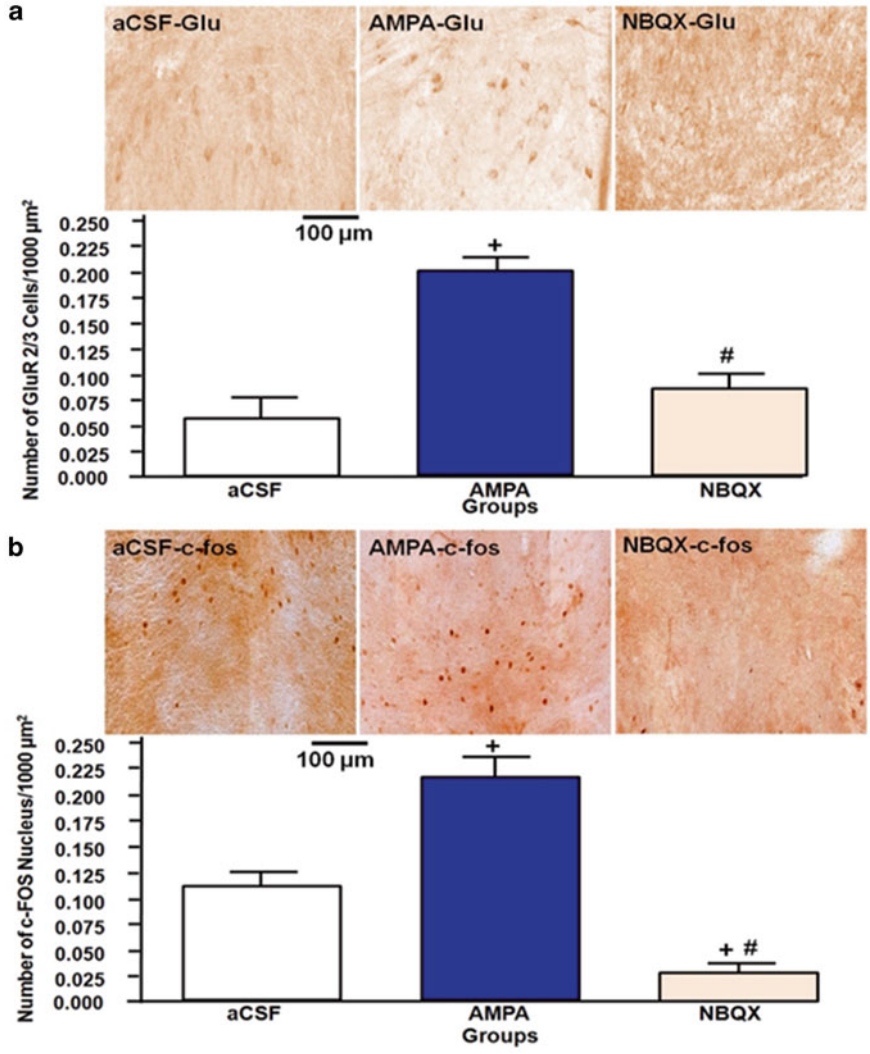

Fig. 34.3 Effects of aCSF, AMPA or NBQX infusions into the cNTS, prior to CChr stimulation on GluR2/3 (Glu) and c-Fos expression in the cNTS (n = 8 per group). (**a**) GluR2/3-labeled cells, (**b**) number of c-Fos labeled cells in the same groups. Data are means of marked cells counted unilaterally ± S.E.M. Plus signs (+) indicate significant difference between aCSF group with their corresponding AMPA or NBQX groups. Pound sign (#) indicates significant difference between AMPA and NBQX groups

values, that decreased from 0.85 ± 0.11 down to 0.72 ± 0.14 µmol/mL a t = 5 min ($p < 0.01$) (Fig. 34.4b). By the contrary, rats injected with NBQX into cNTS 4 min before CChr stimulation, although showed a small increase at 5 and 10 min, it was not significant ($p > 0.05$, ANOVA one way), while the BGR levels decreased at 20 and 30 min (Fig. 34.4b). The comparison between AMPA and NBQX values for BGR did not yield a significant difference ($p > 0.05$, ANOVA).

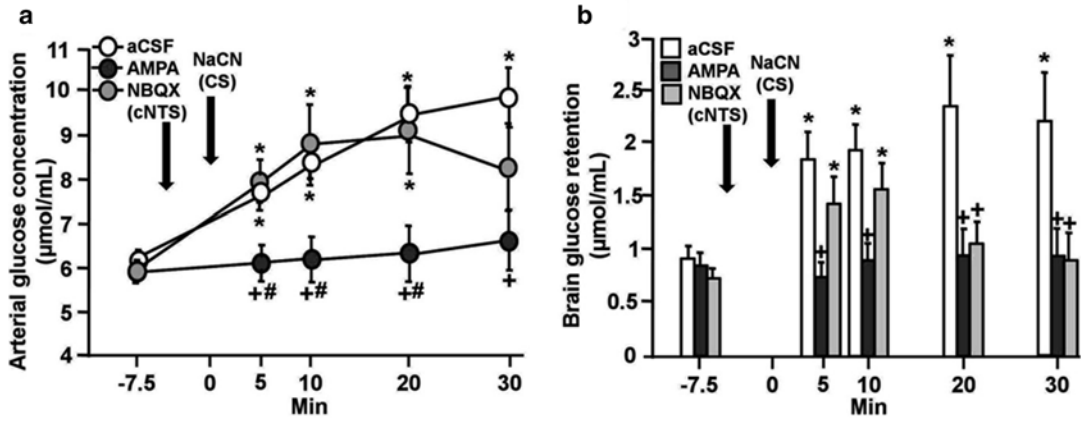

Fig. 34.4 Effects of CChr stimulation 4 min after aCSF (n = 8), AMPA (n = 9) or NBQX (n = 8) infusions in the cNTS of anesthetized rats on: (**a**) arterial glucose concentration, and (**b**) BGR. The values are means ± S.E.M.; $*p < 0.05$ compares with its own basal (Student *t*-test); $+p < 0.05$ compares with aCSF group; $\#p < 0.5$ compares AMPA and NBQX groups (ANOVA)

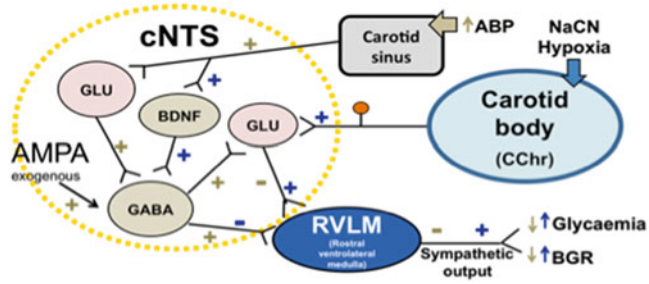

Fig. 34.5 Proposed scheme of cNTS glutamatergic participation on hyperglycemic reflex and brain glucose retention (BGR) elicited by CChr stimulation with NaCN. +, excitation; −, inhibition; *blue* symbols, carotid chemoreceptor pathway; *gray* symbols, carotid baroreceptor pathway

34.4 Discussion

Arterial chemoafferent and baroreceptor neurons share a common development requirements to make their first synapses in the NTS (Brady et al. 1999; Paton et al. 2001). Recent studies in awake rats, provide evidence that the pressor response and sympathoexcitation due to the peripheral chemoreflex are mediated by ionotropic glutamate within the cNTS (Braga et al. 2007). Therefore, we hypothesized a possible involvement of ionotropic glutamate receptors in the effects presented here, after CChr stimulation and AMPA infusion into the cNTS. This study showed that AMPA in the cNTS before CChr stimulation inhibited BGR observed in aCSF group. However, an AMPA/kainate receptor blocker (NBQX) in the cNTS, preceding CChr stimulation, although did not modify the hyperglycemic reflex, significantly inhibited BGR at 20 and 30 min after CChr anoxic stimulation, as observed in aCSF group. The excitatory synaptic transmission in the cardio-respiratory reflexes is predominantly mediated by the AMPA receptors activation in the NTS to undertake a sympathoexcitation reflex (Ozawa et al. 1998; Lin 2009). Furthermore, activation of the AMPA receptor increases the chemoreceptor responses evoked by stimulation in animals exposed to intermittent hypoxia (de Paula et al. 2007), and CChr stimulation activates glutamatergic neurons in the cNTS (Takakura et al. 2006). Activation of AMPA receptors in cNTS induces GABAergic interneuron stimulation with a decrease in sympathetic output, and hyperglycemic reflex-BGR inhibition (Lemus et al. 2008). Sympathetic excitation

stimulates hepatic glycogenolysis and glucagon secretion to provide as much glucose as possible to the brain in a stressful situation (Hoffman 2007). It was previously showed that baroreceptor afferences supply glutamatergic excitatory signals to the NTS, to activate inhibitory GABAergic interneurons in the MVLC (caudal MVL) reducing the sympathetic output in response to increased arterial blood pressure in cardiovascular regulation (Clark et al. 2011). L-glutamate, also mediates the parasympathetic component of the chemoreflex (Braga and Machado 2006), and it could also explain the inhibitory effect of AMPA in the cNTS on glucose variables analyzed in these experiments. The results with NBQX infusion into cNTS indicated that another neurotransmitter might also be participating in the sympathetic component of the reflex studied (Braga and Machado 2006). Our experiments suggest that glutamatergic pathways, via AMPA receptors in the cNTS, may play a role in glucose homeostasis (Fig. 34.5).

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