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Liposome-entrapped GABA modulates the expression of nNOS in NG108-15 cells

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Abstract

Background—Liposomes are concentric lipid vesicles that allow a sustained release of entrapped substances. GABA (γ -aminobutyric acid) is the most prevalent inhibitory neurotransmitter in the central nervous system.

New method—Using GABA-containing liposomes (GL) prepared by the freeze-thawing method, we determined the effect of sustained release of GABA on expression of neuronal nitric oxide synthase (nNOS) and GABA_A receptor (GABA_AR) in an *in vitro* neuronal model.

Results—Neuronal cell line NG108-15 treated with different doses of GL during 24 h showed an increase in expression of GABA_AR (54 and 50% with 10 and 20 ng doses, respectively) and nNOS (138, 157 and 165% with 20, 50 and 100 ng doses, respectively) compared with cells treated with empty liposomes (EL). Additionally, cells treated with 50 ng of GL showed an increase in GABA_AR (23%) after 1h followed by an increase in nNOS (55, 46 and 55%) at 8, 12 and 24h time points, respectively. Immunofluorescence experiments confirmed an increase in nNOS (134%) and basal intracellular levels of nitric oxide (84%) after GL treatment. Further, treatment of cells with GL showed a decrease in expression of protein inhibitor of nNOS (PIN) (26, 66 and 57% with 20, 50 and 100 ng doses respectively) compared with control.

Comparison with existing methods—This is first demonstration for the development of GL that allows sustained slow release of this neurotransmitter.

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Conflict of interest

None.

Disclosures

None.

Conclusion—These results suggest that a slow release of GABA can change the expression of nNOS possibly via alteration in PIN levels in neuronal cells.

Keywords

Liposome; GABA; nNOS; PIN; NG108-15

1. Introduction

Liposomes are spherical vesicles consisting of one or several concentric lipid bilayers that isolate one or more internal aqueous compartments from the external environment (Frezard et al., 2005). These nanostructures can encapsulate hydrophilic or lipophilic substances allowing its sustained release (Batista et al., 2007).

GABA (γ -aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system (CNS) (Beleboni et al., 2004). It exerts its inhibitory effects by acting on two distinct classes of receptors: ionotropic GABA_A and metabotropic GABA_B receptors (Vithlani et al., 2011). GABA_A receptors (GABA_AR) are widely distributed within the mammalian CNS (Palacios et al., 1981). The GABA receptor has a structure where five subunits are arranged around a central pore permeable to Cl⁻ and HCO₃⁻. Much has been learned about GABA and GABA receptors, but the neuronal mechanisms activated after GABAergic stimulation are not fully elucidated, particularly after long-term chronic exposure.

GABA receptors are well known for their important role in modulating behavior and anxiety (Argyropoulos and Nutt, 1999). It is also well known that GABA receptors are implicated in autonomic regulation (Zhang et al., 2002). Imbalance in central GABAergic transmission has been associated with the development and/or progression of various cardiovascular diseases including hypertension (Allen, 2002) and heart failure (Li and Patel, 2003).

In this regard, we have recently reported that rats treated with GABA-containing liposomes (GL) into the lateral cerebral ventricles demonstrated attenuated response of renal sympathetic nerve activity (RSNA) to bicuculline methiodide, a powerful GABA_AR receptor antagonist (Vaz et al., 2015). Animals that received GL intracerebroventricular also exhibited reduced anxiety and blunted tachycardia in the acute emotional stress trial. These findings indicated that GL could be a potential tool for neuroscience studies requiring the slow release of GABA within the CNS (Vaz et al., 2015). However, the neuronal mechanism/s that are altered as a result of long-term GABA release from GL are not entirely clear.

Nitric oxide (NO) is a gaseous molecule that works as an atypical neurotransmitter (Schuman and Madison, 1994), acting as an important mediator of intracellular signaling in various tissues, including the CNS. It is synthesized from L-arginine by the enzyme NO synthase (NOS). There are at least three different forms of NOS, but in the CNS, the prevalent form is neuronal NOS (nNOS) (Calabrese et al., 2007). This enzyme is regulated by multiple molecular mechanisms including the regulation by Ca²⁺-calmodulin and by the protein inhibitor of nNOS (PIN). While the increase of intracellular calcium stimulate the nNOS production (Szabo, 1996), increased, PIN expression causes inhibition of nNOS

(Sharma et al., 2013) by preventing the formation of the catalytic active dimeric form of nNOS.

To understand the efficacy of GL as a new tool for the chronic release of GABA, in the present study, we tested the hypothesis that the sustained release of GABA encapsulated into liposomes could influence the expression of GABA_AR and expression of nNOS in cultured neuronal cell line NG108-15. This examination of the interaction between GABA and nNOS could shed light on the possible mechanism/s by which long-term GABAergic stimulation could result in physiological changes.

2. Material and Methods

2.1. Lipids

GABA was obtained from Sigma. L- α -distearoyl-phosphatidylcholine (DSPC), cholesterol (CHOL) and distearoyl-phosphatidylethanolamine-polyethylene glycol 2000 (DSPE-PEG2000) were obtained from Lipoid.

2.2. Preparation of liposomal formulations

Liposomal formulations were prepared according to the method of Vaz et al. (2015). The vesicles were made from the lipids DSPC, CHOL, and DSPE-PEG2000 at 5:4:0.3 molar ratios. GABA-containing liposomes (GL) and empty liposomes (EL) were prepared by the freeze-thawing method as first described by (Mayer et al., 1985), followed by calibration of vesicle size. Briefly, the lipids were co-dissolved in chloroform and placed in a rotary evaporator apparatus (bath 60°C) to remove the organic solvent and form a lipid film. The film was then hydrated with GABA solution (0.3M in 0.9% NaCl) and saline (0.9% NaCl) for GL and EL, respectively, at a final lipid concentration of 100 g/L. The resulting multilamellar vesicles were submitted to 5 cycles of freezing-thawing, leading to the formation of freeze-thawed multilamellar vesicles (FATMLVs) (Mayer et al., 1985). After this, FATMLVs were repeatedly extruded at 65°C through two stacked polycarbonate membranes of 200-nm pore size, leading to the formation of calibrated unilamellar vesicles (Nayar et al., 1989). The suspension was finally submitted to dialysis (MWCO 12,000 Da) against saline (0.9% NaCl) to remove non-encapsulated GABA (Figure 1). The mean hydrodynamic diameter and polydispersity index of the vesicles were determined by photon correlation spectroscopy (Malvern Zetasizer Nano ZS90; Malvern Instruments, United Kingdom).

2.3. Incorporation efficiency of GABA into liposomes

GABA content was determined by exploiting its specific interaction with fluorescamine as previously described by Udenfriend et al. (1972) with following modifications. Briefly, 5 μ L of GL was added in a 5 mL of the assay solution of methanol (0.5 ml), water (1.5 ml), borate buffer (20 mM, pH 8.2, 2.5 ml) and fluorescamine (0.3 mg/ml diluted in acetone). The amount of encapsulated GABA was determined using the fluorescence of GABA-fluorescamine complex at the maximum excitation of 388 nm and maximum emission at 486 nm. A calibration curve in the presence of EL, but known amounts of GABA, was constructed and used.

2.4. Cell culture

In vitro experiments were performed using NG-108-15 cells (neuroblastoma X glioma) purchased from American Type Culture Collection (ATCC HB-12317). Cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin G, and streptomycin in a humidified atmosphere of 5% CO₂ at 37°C.

2.5. Experimental protocols

The cultured neuronal NG108 cells were treated in two different ways: i) Neurons were treated in a dose- dependent manner with doses of GL (5, 10, 20, 50, 100 ng), a control group was treated with 5 µl of EL. The cells were harvested after 24 hours of treatment. ii) In the second experiment, neurons were treated with 50 ng of GL or GABA solution (GS – 50 ng) and harvested at different time points (0, 1, 2, 4 8, 12 and 24 hours) after treatment. Immunostaining experiments and NO measurement were performed after 12 hours of treatment with 50 ng of GL.

2.6. Measurement of nNOS, GABA_AR, CREB and PIN protein expression

Western blotting was performed to determine protein expression of nNOS, GABA_AR and PIN in NG108 cells lysates as previously reported (Sharma et al., 2013). Cells were washed three times with ice-cold PBS and lysed in protein extraction buffer (10 mM Tris, 1 mM EDTA, 1% SDS, 0.1% Triton X-100) supplemented with protease inhibitor cocktail. The protein content of lysate was estimated using the bicinchoninic acid method with bovine serum albumin as standard (Pierce, Rockford, IL). Protein lysates (20–30µg) were electrophoresed on 10% sodium dodecyl sulfatepolyacrylamide gel in Tris-glycine running buffer and transferred to a polyvinylidene difluoride membrane (Millipore). Nonspecific binding sites were blocked by incubating the membrane with 5% nonfat dry milk (wt/vol) in TBST buffer (10 mM Tris, 150 mM NaCl, 0.05% Tween 20) at ambient temperature for 1 hour. The membrane was probed with primary antibodies for nNOS (sc 5302, Santa Cruz Biotechnology), GABA_AR (AB10532, Millipore), PIN (sc13969, Santa Cruz Biotechnology), GAPDH (sc25778, Santa Cruz Biotechnology), CREB (Cell Signaling, #9104), CREB-P (cell signaling, #9198) and β-actin (sc-47778, Santa Cruz Biotechnology) at 4°C overnight followed by incubation with a corresponding peroxidase-conjugated secondary antibody. The signals were visualized using an enhanced chemiluminescence substrate (Pierce Chemical) and detected by Work lab digital image system. The net intensity of the respective protein bands was then expressed as a ratio to the net intensity of their respective housekeeping GAPDH or β-actin bands.

2.7. Immunofluorescent staining of GABA_AR and nNOS

Immunofluorescence staining was performed for nNOS and GABA_AR in NG108 cells as described previously (Sharma et al., 2013; Sharma et al., 2012; Zheng et al., 2011). Briefly, adherent NG108 cells were grown on laminin-coated 6 mm Transwel-Clear™ inserts (Corning, Costar). Cells were fixed in 4% paraformaldehyde for 10 min and then permeabilized with 0.2% Triton X-100 for 20–30 min. The incubation was done with GABA_AR (AB10532, Millipore), and nNOS (sc-302, Santa Cruz Biotechnology) primary

antibody for 4–6 hours at 4°C. After washing with PBS, the cells were incubated with Cy3-conjugated goat anti-rabbit (1:500) and Cy2-conjugated goat anti-mouse secondary antibody (1:500) for 4 hours in dark at room temperature. The slides were observed under a Leica-DMR microscope with corresponding filters, scaled and analyzed by ImageJ software (NIH, USA).

2.8. Intracellular NO measurement

Intracellular NO concentration was measured in NG108 cells treated with empty liposomes (EL) or GL for 12 hours using 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM) fluorescence dye. Sub-confluent NG108 cells were grown in a Nunc Lab-Tek chamber before experimentation. Briefly, cells were loaded with DAF-AM (5 μ M) for 30 min at 37°C followed by additional incubation after washing for 20 min to complete de-esterification of the dye. NO concentration was measured before and after stimulation with glutamate (10 μ M) by DAF-FM fluorescence using a Zeiss LSM 510 META confocal microscope with an $\times$ 63 oil immersion objective and 488 nm wavelength excitation.

2.9. Silencing of PIN

For this experiment, siRNA targeting PIN and its negative control were purchased from Invitrogen (Carlsbad, CA) Transient transfection was performed in NG108 cells (2×10^5 cells) cultured in six-well plates using Lipofectamine 2000 (Invitrogen) according to the manufacturer instructions. After 4 hours incubation with siRNA-lipid, the cells were re-plated into the complete growth medium with FBS. Forty-eight hours post-transfection, cells media was changed and cells were treated with GL or EL for further experiments,

2.10. Statistical analysis

Data was analyzed by Student's *t*-tests (unpaired) and One Way ANOVA. For all statistical analysis, the critical *P* value was considered < 0.05. Data are shown as mean $\pm$ SEM.

3. Results

3.1. Characterization of the preparation of liposome-encapsulated GABA

Table 1 shows the major characteristic of liposomal preparation. The encapsulation of GABA was achieved with an efficiency of 35% and a ratio of encapsulated GABA to lipid (GABA/lipid) of 0.093 (w/w). The resulting liposomal formulations showed mean hydrodynamic vesicle diameters of 175 nm for EL and 224 nm for GL, with polydispersity indexes below 0.2 for both preparations, indicative of monodisperse vesicle suspensions.

3.2. Effect of liposome-encapsulated GABA on expression of GABA_AR and nNOS in NG108 cells

NG108-15 cells were treated with different doses of GL for 24 hours as shown in Figure 2A. An increased expression of GABA_AR of approximately 54 and 50% was observed at doses 10 and 20 ng respectively when compared with EL treated control cells. In contrast, to GABA_AR expression, nNOS was increased at higher doses, which were 138, 157 and 165% with 20, 50 and 100 ng respectively, when compared with cells treated with EL (Figure 2 B).

In the time course experiments, NG108-15 cells treated with the same amount of GL (50 ng) and harvested at different time points (0, 1, 2, 4, 8, 12 and 24 hours), showed an increase (23%) of GABA_AR at early time points of exposure (1 hour after treatment) and decrease after 24 hours (Figure 2C). On the other hand, nNOS expression increased (55, 46 and 55%) at 8, 12 and 24 hours' time points after treatment with GL (Figure 2D). In complementary control experiments, we observed that NG108 cells treated with free GABA solution showed a significant increase of nNOS expression (48 and 65%) at 8 and 12-hour time points respectively (Figure 3). Moreover, immunostaining results suggest that 12 hours of exposure to GL in NG108-15 cells resulted in marked increase in expression of nNOS without demonstrating any changes in GABA_AR receptor expression (Figure 4A) when compared to cells treated with EL. While quantitative analysis showed a 134 % increase in nNOS (Figure 2B), there was no significant increase in GABA_AR expression (Figure 4C).

3.3 Effect of liposome-encapsulated GABA on NO levels in NG108 cells

Additionally, confocal images showed that cells treated with GL demonstrated an increase (84%) in baseline levels of NO when compared with control cells. Further, after stimulation with glutamate, NG-108 cells treated with EL showed the increase (41%) in intracellular NO, however, this effect was not observed in cells treated with GL (Figure 5) suggesting that the basal levels of NO in these cells were already high before the treatment with glutamate.

3.4. Effect of liposome-encapsulated GABA on expression of PIN in NG108 cells

To determine the underlying mechanism/s that may be involved in the changes observed in nNOS evoked by GABA, we measured the expression of CREB phosphorylated (CREB-P) and CREB in samples treated with GL. As shown in Figure 6, CREB and CREB-P expression were not altered in cells treated with GL, in both dose dependent (A) and time course (B) experiments, suggesting that perhaps CREB activation may not be involved in the increased nNOS expression in response to GL treatment. As another alternative since PIN is an endogenous inhibitor for nNOS, we reasoned that GL was maybe involved in changing the PIN expression and thus nNOS. Figure 7 shows that PIN expression was decreased (26, 66 and 57%) in NG108 cells treated with increasing amounts of GL (20, 50 and 100 ng) (Figure 7A) and (28 and 53%) over the 12 and 24-hour time points (Figure 7 B), respectively, after treatment.

In subsequent experiments, we observed that silencing PIN in these cells resulted in a 60% decrease in the expression of PIN compared to vehicle controls. Interestingly treatment with EL or GL showed similar results i.e. 77 and 65% decrease in PIN expression, respectively. Consistent with our hypothesis, silencing of PIN resulted in a concomitant increase (50%) in nNOS expression. Further, the expression of nNOS was increased in all groups independent of cells treatment that showed a decrease in expression of PIN and also there were no significant differences between groups (Figure 8 A, B).

4. Discussion

The major findings of this study are that the treatment of NG108-15 with GL was able to increase the levels of GABA_AR with moderately low dose, and the response was relatively

early within the first hour of exposure. GL also induced an increase in expression of nNOS; however, this happened after exposure to higher concentrations and longer duration of treatment with GL. Consistent with these observations, GL exposure also induced an increase in intracellular NO. Furthermore, it appears that PIN may be part of the mechanism by which GABA changes nNOS expression.

Liposomes represent an advanced technology to deliver active molecules to the site of action. The analyses of the formulations evolve consistently in recent years, and many of them are in clinical use (Immordino et al., 2006). Recent studies have demonstrated the potential use of liposomes to access the brain through an intranasal pathway. This route of administration is of interest since the drug/s can be directly delivered to the brain aptly crossing the blood-brain barrier (BBB) (Kozlovskaya et al., 2014). Utilizing this mode of carrier mechanism, various studies have used administration of intranasal liposomes in many diseases, such as Alzheimer (Corace et al., 2014; Zheng et al., 2015), Parkinson (Migliore et al., 2014) and tuberculosis (Leemans et al., 2001). Also, intranasal liposomes have also been used in the preparation of vaccines against bacterial and fungal infectious diseases such as pneumonia, influenza, and tuberculosis (Khatri et al., 2008; Romero and Morilla, 2011; Tada et al., 2015; Tiwari et al., 2011).

The encapsulation of GABA in lipid vesicles was evaluated previously by Loeb group (Loeb 1982, 1986). They hypothesized that the liposomal carrier might enhance the penetration of GABA across the blood brain barrier and reported a reduction in epileptic activity after the intraperitoneal administration of GABA encapsulated in liposomes. However, it is important to note that Loeb's group in their studies, used a different encapsulation method utilizing only the natural lipid phosphatidylserine for the preparation of liposomes. Liposomes prepared with phosphatidylserine are expected to be less stable and have leaky vesicles compared to those used in the current preparation. In the present study, liposome was prepared with the high-phase transition temperature lipid phosphatidylcholine (DSPC) and cholesterol, to achieve a very low membrane permeability (Silva-Barcellos et al., 2001). Accordingly, *in vivo* release study, with this same preparation (Vaz et al., 2015) indicated a sustained release of GABA from liposomes, with only ~60% of GABA release after 5 days of incubation at 37°C. Other important characteristics of these liposomes are their mean diameter of 200 nm and the incorporation of a pegylated lipid, to slow down their capture by cells for endocytosis/phagocytosis and the cell-mediated release of encapsulated drug (Frezard et al., 2011). However, the early response after exposure of NG108-15 to GL suggests that liposome capture by these cells takes place and exerts a significant effect on the proteins measured in this study.

All these characteristics make liposomes an important tool to study the effect of slow release of GABA in the central and/or peripheral nervous system. Previous studies by Vaz et al. (2015) showed that the intracerebroventricular microinjection of the same liposomal preparation evoked attenuated response in renal sympathetic nerve activity to microinjection of bicuculline methiodide. Additionally, these rats treated with GL presented blunted tachycardia in stress trials and showed reduced anxiety as revealed by elevated plus maze suggesting a functional change in the neuronal circuitry involved in these responses.

The neuroblastoma X glioma hybrid NG108-15 cell line (Hamprecht, 1977) demonstrates neuronal properties and has been extensively used as an in-vitro neuronal model (Cargill and Thibault, 1996; Chemin et al., 2002). NG108-15 express both GABA_AR (Searles and Singer, 1988) and nNOS (Sharma et al., 2011) and thus represent an ideal neuronal cell line to study the interaction between these two mediators. In the present study, we used this cellular model to elucidate the possible molecular pathways involved in the downstream effects of GABA exposure on neurons. There is ample evidence to suggest the importance of GABA and NO in the regulation of sympathetic outflow and consequent cardiovascular effects (Allen, 2002; Li et al., 2003; Zhang and Patel, 1998). Imbalance in central GABAergic and nitrergic transmission has been associated with the development of cardiovascular diseases including hypertension (Allen, 2002) and heart failure (Li and Patel, 2003).

Based on our previous study (Vaz et al., 2015) we speculate that encapsulation of GABA in liposomes allow the slow release GABA to the cells in the media. The results show that smaller amounts and shorter time exposure to GABA were able to increase the GABA_AR expression. At higher doses or using a longer-term treatment, GL was able to change the expression of nNOS and potentially increase the availability of NO in the theses cells. Taking into consideration the concentration dependence and time course of the changes observed in GABA_AR and nNOS, we speculate that a prior activation of GABA_AR could evoke intracellular alterations that are possibly responsible for an increase in nNOS expression. As to specific molecular mechanism that may be involved remain to be examined. Cells incubated with free GABA showed an increase in nNOS only at 8 and 12-hour time points, unlike cells treated with GL that demonstrated an increase in nNOS at 8, 12 and 24-hour time points (Figure 2). The slow release of GABA through GL was likely able to increase nNOS even after 24 hours of incubation indicating the importance of utilization of liposomes for slow release.

Current evidence indicates that, in the CNS, NO is produced enzymatically in postsynaptic structures in response to activation of excitatory amino acid receptors such as glutamatergic NMDA receptors (Garthwaite, 1991). Thus, stimulation of the cells with glutamate increases intracellular NO. In the present study, in addition to increased expression and staining nNOS in NG108-15 cells treated with GL, we also examined the increase in intracellular NO in response to glutamate exposure. When control cells were stimulated with glutamate, they showed an increase in intracellular levels of NO; the same effect was not observed in cells treated with GL. This lack of response in GL-treated cells was probably because the baseline concentration of NO in these cells was already relatively high before the treatment with glutamate. So it is possible that additional stimulation was not able to show a further increase in intracellular NO concentration.

In the adult mammalian brain GABA functions as an inhibitory neurotransmitter, however in the early stages of development including the embryonic stage, GABA is the primary excitatory neurotransmitter (Ben-Ari, 2002; Owens and Kriegstein, 2002). Experiments performed in cortex slices of 5 days old rats showed that GABA_AR activation leads to the opening of L-type voltage-gated calcium channels resulting in an increase in Ca²⁺ influx which in turn results in the phosphorylation and activation of CREB. The phosphorylated CREB (CREB-P) can induce the increase in nNOS (Mantelas et al., 2003). However, in our

preliminary studies, CREB-P was not changed in time course or dose-dependent experiments.

Another possibility that could evoke change in the nNOS expression would be an alteration in PIN expression, which is known to be regulating nNOS expression via destabilizing catalytically active dimeric nNOS. In the present study, we observed that PIN was decreased with the GL treatment in dose-dependent and time course manner (Figure 7). Taken together these results suggest that one contributing mechanism to explain how GABA changes nNOS expression maybe via alterations in PIN expression. However, the specific details of this possible pathway and their ramifications remain to be explored further.

In conclusion, this study showed the feasibility of GL as the viable new tool for influencing GABA neurotransmission in neuronal cells and thus potentially within the CNS.

Furthermore, these results demonstrate that sustained administration of GABA can alter NO availability in neuronal populations that have the machinery to make NO such as the NG-108-15 cells.

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References

1. Allen AM. Inhibition of the hypothalamic paraventricular nucleus in spontaneously hypertensive rats dramatically reduces sympathetic vasomotor tone. *Hypertension*. 2002; 39:275–280. [PubMed: 11847197]
2. Argyropoulos SV, Nutt DJ. The use of benzodiazepines in anxiety and other disorders. *European neuropsychopharmacology : the journal of the European College of Neuropsychopharmacology*. 1999; 9(Suppl 6):S407–S412. [PubMed: 10622687]
3. Batista CM, Carvalho CMBd, Magalhães NSS. Lipossomas e suas aplicações terapêuticas: Estado da arte. *Brazilian Journal of Pharmaceutical Sciences*. 2007
4. Belebony RO, Carolino RO, Pizzo AB, Castellan-Baldan L, Coutinho-Netto J, dos Santos WF, Coimbra NC. Pharmacological and biochemical aspects of GABAergic neurotransmission: pathological and neuropsychobiological relationships. *Cell Mol Neurobiol*. 2004; 24:707–728. [PubMed: 15672674]
5. Ben-Ari Y. Excitatory actions of gaba during development: the nature of the nurture. *Nature reviews. Neuroscience*. 2002; 3:728–739. [PubMed: 12209121]
6. Calabrese V, Mancuso C, Calvani M, Rizzarelli E, Butterfield DA, Stella AM. Nitric oxide in the central nervous system: neuroprotection versus neurotoxicity. *Nature reviews. Neuroscience*. 2007; 8:766–775. [PubMed: 17882254]
7. Cargill RS 2nd, Thibault LE. Acute alterations in $[Ca^{2+}]_i$ in NG108-15 cells subjected to high strain rate deformation and chemical hypoxia: an in vitro model for neural trauma. *Journal of neurotrauma*. 1996; 13:395–407. [PubMed: 8863195]
8. Chemin J, Nargeot J, Lory P. Neuronal T-type α 1H calcium channels induce neuriteogenesis and expression of high-voltage-activated calcium channels in the NG108-15 cell line. *J Neurosci*. 2002; 22:6856–6862. [PubMed: 12177183]

9. Corace G, Angeloni C, Malaguti M, Hrelia S, Stein PC, Brandl M, Gotti R, Luppi B. Multifunctional liposomes for nasal delivery of the anti-Alzheimer drug tacrine hydrochloride. *Journal of liposome research*. 2014; 24:323–335. [PubMed: 24807822]
10. Frezard F, dos Santos RA, Fontes MA. Liposome-encapsulated neuropeptides for site-specific microinjection. *Methods Mol Biol*. 2011; 789:343–355. [PubMed: 21922420]
11. Frezard F, Schettini DA, Rocha OG, Demicheli C. Lipossomas: propriedades físico-farmacológicas, aplicações à base de antimônio. *Química Nova*. 2005; 28:511–518.
12. Garthwaite J. Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends in neurosciences*. 1991; 14:60–67. [PubMed: 1708538]
13. Hamprecht B. Structural, electrophysiological, biochemical, and pharmacological properties of neuroblastoma-glioma cell hybrids in cell culture. *International review of cytology*. 1977; 49:99–170. [PubMed: 16829]
14. Immordino ML, Dosio F, Cattel L. Stealth liposomes: review of the basic science, rationale, and clinical applications, existing and potential. *Int J Nanomedicine*. 2006; 1:297–315. [PubMed: 17717971]
15. Khatri K, Goyal AK, Gupta PN, Mishra N, Mehta A, Vyas SP. Surface modified liposomes for nasal delivery of DNA vaccine. *Vaccine*. 2008; 26:2225–2233. [PubMed: 18396362]
16. Kozlovskaya L, Abou-Kaoud M, Stepensky D. Quantitative analysis of drug delivery to the brain via nasal route. *J Control Release*. 2014; 189:133–140. [PubMed: 24997277]
17. Leemans JC, Juffermans NP, Florquin S, van Rooijen N, Vervoordeldonk MJ, Verbon A, van Deventer SJ, van der Poll T. Depletion of alveolar macrophages exerts protective effects in pulmonary tuberculosis in mice. *J Immunol*. 2001; 166:4604–4611. [PubMed: 11254718]
18. Li Y, Zhang W, Stern JE. Nitric oxide inhibits the firing activity of hypothalamic paraventricular neurons that innervate the medulla oblongata: role of GABA. *Neuroscience*. 2003; 118:585–601. [PubMed: 12710969]
19. Li YF, Patel KP. Paraventricular nucleus of the hypothalamus and elevated sympathetic activity in heart failure: the altered inhibitory mechanisms. *Acta physiologica Scandinavica*. 2003; 177:17–26. [PubMed: 12492775]
20. Mantelas A, Stamatakis A, Kazanis I, Philippidis H, Stylianopoulou F. Control of neuronal nitric oxide synthase and brain-derived neurotrophic factor levels by GABA-A receptors in the developing rat cortex. *Brain research. Developmental brain research*. 2003; 145:185–195. [PubMed: 14604759]
21. Mayer LD, Hope MJ, Cullis PR, Janoff AS. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. *Biochimica et biophysica acta*. 1985; 817:193–196. [PubMed: 4005257]
22. Migliore MM, Ortiz R, Dye S, Campbell RB, Amiji MM, Waszczak BL. Neurotrophic and neuroprotective efficacy of intranasal GDNF in a rat model of Parkinson's disease. *Neuroscience*. 2014; 274:11–23. [PubMed: 24845869]
23. Nayar R, Hope MJ, Cullis PR. Generation of large unilamellar vesicles from long-chain saturated phosphatidylcholines by extrusion technique. *Biochim. Biophys. Acta*. 1989; 986:200–206.
24. Owens DF, Kriegstein AR. Is there more to GABA than synaptic inhibition? *Nature reviews. Neuroscience*. 2002; 3:715–727. [PubMed: 12209120]
25. Palacios JM, Wamsley JK, Kuhar MJ. High affinity GABA receptors-autoradiographic localization. *Brain Res*. 1981; 222:285–307. [PubMed: 6269695]
26. Romero EL, Morilla MJ. Topical and mucosal liposomes for vaccine delivery. *Wiley interdisciplinary reviews. Nanomedicine and nanobiotechnology*. 2011; 3:356–375. [PubMed: 21360692]
27. Schuman EM, Madison DV. Nitric oxide and synaptic function. *Annual review of neuroscience*. 1994; 17:153–183.
28. Searles CD, Singer HS. The identification and characterization of a GABAergic system in the cholinergic neuroblastoma × glioma hybrid clone NG108-15. *Brain Res*. 1988; 448:373–376. [PubMed: 3378161]

29. Sharma NM, Llewellyn TL, Zheng H, Patel KP. Angiotensin II-mediated posttranslational modification of nNOS in the PVN of rats with CHF: role for PIN. *Am J Physiol Heart Circ Physiol*. 2013; 305:H843–H855. [PubMed: 23832698]
30. Sharma NM, Zheng H, Li YF, Patel KP. Nitric oxide inhibits the expression of AT1 receptors in neurons. *American journal of physiology. Cell physiology*. 2012; 302:C1162–C1173. [PubMed: 22218225]
31. Sharma NM, Zheng H, Mehta PP, Li YF, Patel KP. Decreased nNOS in the PVN leads to increased sympathoexcitation in chronic heart failure: role for CAPON and Ang II. *Cardiovasc Res*. 2011; 92:348–357. [PubMed: 21831995]
32. Silva-Barcellos NM, Frezard F, Caligiorne S, Santos RA. Long-lasting cardiovascular effects of liposome-entrapped angiotensin-(1–7) at the rostral ventrolateral medulla. *Hypertension*. 2001; 38:1266–1271. [PubMed: 11751701]
33. Szabo C. Physiological and pathophysiological roles of nitric oxide in the central nervous system. *Brain Res Bull*. 1996; 41:131–141. [PubMed: 8886382]
34. Tada R, Hidaka A, Iwase N, Takahashi S, Yamakita Y, Iwata T, Muto S, Sato E, Takayama N, Honjo E, Kiyono H, Kunisawa J, Aramaki Y. Intranasal Immunization with DOTAP Cationic Liposomes Combined with DC-Cholesterol Induces Potent Antigen-Specific Mucosal and Systemic Immune Responses in Mice. *PloS one*. 2015; 10:e0139785. [PubMed: 26440657]
35. Tiwari B, Agarwal A, Kharya AK, Lariya N, Saraogi G, Agrawal H, Agrawal GP. Immunoglobulin immobilized liposomal constructs for transmucosal vaccination through nasal route. *Journal of liposome research*. 2011; 21:181–193. [PubMed: 20626315]
36. Udenfriend S, Stein S, Bohlen P, Dairman W, Leimgruber W, Weigle M. Fluorescamine: a reagent for assay of amino acids, peptides, proteins, and primary amines in the picomole range. *Science*. 1972; 178:871–872. [PubMed: 5085985]
37. Vaz GC, Bahia AP, de Figueiredo Muller-Ribeiro FC, Xavier CH, Patel KP, Santos RA, Moreira FA, Frezard F, Fontes MA. Cardiovascular and behavioral effects produced by administration of liposome-entrapped GABA into the rat central nervous system. *Neuroscience*. 2015; 285:60–69. [PubMed: 25446344]
38. Vithlani M, Terunuma M, Moss SJ. The dynamic modulation of GABA(A) receptor trafficking and its role in regulating the plasticity of inhibitory synapses. *Physiological reviews*. 2011; 91:1009–1022. [PubMed: 21742794]
39. Zhang K, Li YF, Patel KP. Reduced endogenous GABA-mediated inhibition in the PVN on renal nerve discharge in rats with heart failure. *Am J Physiol Regul Integr Comp Physiol*. 2002; 282:R1006–R1015. [PubMed: 11893604]
40. Zhang K, Patel KP. Effect of nitric oxide within the paraventricular nucleus on renal sympathetic nerve discharge: role of GABA. *Am J Physiol*. 1998; 275:R728–R734. [PubMed: 9728069]
41. Zheng H, Liu X, Patel KP. Angiotensin-converting enzyme 2 overexpression improves central nitric oxide-mediated sympathetic outflow in chronic heart failure. *Am J Physiol Heart Circ Physiol*. 2011; 301:H2402–H2412. [PubMed: 21963832]
42. Zheng X, Shao X, Zhang C, Tan Y, Liu Q, Wan X, Zhang Q, Xu S, Jiang X. Intranasal H102 Peptide-Loaded Liposomes for Brain Delivery to Treat Alzheimer's Disease. *Pharmaceutical research*. 2015; 32:3837–3849. [PubMed: 26113236]

Highlights

1. GABA (γ -aminobutyric acid) -containing liposomes (GL) show the slow release of GABA.
2. Cells treated with GL showed an increase in neuronal nitric oxide synthase expression and intracellular nitric oxide.
3. The increase in nNOS expression was accompanied by a decrease in protein inhibitor of nNOS (PIN) expression.
4. Released GABA may cause activation in GABA_A receptors.
5. Alterations in PIN expression may be a consequence of GABA_A receptor activation.

Liposomes preparation

Figure 1.
Schematic drawing of the preparation of liposomes

Figure 2.

(A) Protein expression of GABA_AR with increasing concentrations of GL treatment. *Top*: a representative gel. *Bottom*: densitometry analyses of GABA_AR protein levels normalized to GAPDH. (B) Protein expression of nNOS in NG108 cells with increasing concentrations of GL treatment. *Top*: a representative gel. *Bottom*: densitometry analyses of nNOS protein levels normalized to GAPDH. (C) Protein expression of GABA_AR in NG108 cells treated with 50 ng of GL and harvested at different times. *Top*: a representative gel. *Bottom*: densitometry analyses of GABA_AR protein levels normalized to GAPDH. (D) Protein expression of nNOS in NG108 cells treated with 50 ng of GL and harvests in different times. *Top*: a representative gel. *Bottom*: densitometry analyses of nNOS protein levels normalized to GAPDH. *P < 0.05 different from control (EL or time 0). Values were mean ± SE from four Independent experiments.

Figure 3.

Protein expression of nNOS treated with 50 ng of GABA saline (0.9%) solution and harvested at different times. *Top*: a representative gel. *Bottom*: densitometry analyses of nNOS protein levels normalized to actin. Values were mean ± SE from five Independent experiments *P<0.05 Compared to control without treatment.

A **B** **C** **Figure 4.**

Immunofluorescence of the NG108 cells stained for nNOS (green) and GABA_AR (red) after treatment with EL and GL for 12h. (A) Photomicrographs showing Immunofluorescence for nNOS, GABA_AR and merge in EL and GL-treated cells. (B and C) Quantification of fluorescent images for nNOS and GABA_AR. Values are mean $\pm$ SEM of four different experimental images *P < 0.05 vs. EL.

Figure 5. Confocal photomicrographs of intracellular nitric oxide NO staining with DAF-FM in NG108 cells treated with EL (A) or GL (B) for 12h before and after 10 μ M glutamate stimulation. C: Cumulative data were showing the baseline of intracellular NO and after stimulation with glutamate (n = 8–10 cells from three cover slips in each group). *P < 0.05 different from EL basal.

Figure 6.

(A) Protein expression of CREB and CREB-P in NG108 cells treated with increasing concentrations of GL. *Top*: representative gels. *Bottom*: A graph showing the ratio of CREB to CREB-P. (B) Protein expression of CREB and CREB-P in NG108 cells treated with 50 ng of GL and harvested at different time points. *Top*: a representative gel. *Bottom*: densitometry analyses of CREB to CREB-P protein levels. Values were mean $\pm$ SE from four Independent experiments.

Figure 7.

(A) Protein expression of PIN in NG108 cells treated with increasing concentrations of GL. *Top*: a representative gel. *Bottom*: densitometry analyses of PIN protein levels normalized to GAPDH. (B) Protein expression of PIN in NG108 cell line treated with 50 ng of GL and harvests in different times. *Top*: a representative gel. *Bottom*: densitometry analyses of PIN protein levels normalized to GAPDH. Values were mean $\pm$ SE from four to five Independent experiments. *P < 0.05 different from control.

Figure 8.

(A) Protein expression of PIN in NG108 cells control, knockdown (KD) for PIN (PIN siRNA), KD treated with empty liposomes (PIN siRNA-EL) and KD treated with GABA liposome (PIN siRNA-GL). *Top*: a representative gel. *Bottom*: densitometry analyses of PIN protein levels normalized to GAPDH. (B) Protein expression of neuronal nitric oxide (nNOS) in control, PIN siRNA, PIN siRNA-EL and PIN siRNA-GL. *Top*: a representative gel. *Bottom*: densitometry analyses of nNOS protein levels normalized to GAPDH. Values were mean $\pm$ SE from four Independent experiments *P<0.05 Compared to scramble siRNA. *P < 0.05 vs. control.

Table 1

Characteristics of liposomes preparation

Characteristics	EL	GL
Encapsulated solution	Saline (0.9% NaCl)	GABA (0.3M in 0.9% NaCl)
Encapsulation efficiency (%)	-	35
Size (nm)	175 ± 7	191 ± 8
Polidispersity Index	0.111 ± 0.024	0.148 ± 0.032

EL: empty liposomes (n = 4)

GL : GABA liposomes (n = 6)