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SHORT COMMUNICATION

Decreased levels of GABA in the inferior colliculus of the epilepsy-prone hamster (GPG/Vall)

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Summary Abnormalities in GABA levels in the central nucleus of the inferior colliculus (CNIC) of the epilepsy-prone hamster (GPG/Vall) were evaluated by using immunohistochemistry, densitometry and high performance liquid chromatography (HPLC). These findings demonstrate a decrease both in GABA immunostaining (neuropil and neurons) and in GABA concentration (HPLC) in the CNIC of the epileptic hamster compared to control animals. These decreases may reflect a reduced availability of this neurotransmitter that may act as an audiogenic seizure-initiating factor.

Introduction

The contribution of the inferior colliculus (IC) in the neuronal network for audiogenic seizures (AGS) has been evaluated in a variety of audiogenic seizure-susceptible animals (Faingold, 1999). In particular, the central nucleus of the inferior colliculus (CNIC), which receives information from different cell types in the auditory brainstem, has been proposed to be the initiation site for AGS (Faingold et al., 1986). Accordingly, disturbances of calcium-buffering mechanisms and also abnormalities in the expression of neurotransmitter and/or their receptors occur in CNIC of several genetic models of AGS (Fuentes-Santamaría et al., 2005; Lasley, 1991). A deficiency of the inhibitory neurotransmitter GABA in the IC has been suggested to be one of the most important mechanisms modulating AGS (Faingold, 2002). Accordingly, the present study investigates possible abnormalities in the levels of GABA in the CNIC of the GPG/Vall, an endogamic strain of Golden Syrian hamsters (*Mesocricetus auratus*) that exhibits generalized tonic–clonic seizures in response to sound stimulation (Soria-Milla et al., 1987; Fuentes-Santamaría et al., 2005, 2007), by using immunohistochemistry, densitometric analysis and HPLC.

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Methods

Syrian Golden hamsters (control and GPG/Vall) were handled according to the Guidelines of the European Communities Council Directive (DOCE L222; 8-24-1999) and the Spanish legislation for the use and care of laboratory animals (RD 233/88). All the animals used for immunocytochemistry and for HPLC were tested for AGS susceptibility. Because these seizures first appear around day 20 of postnatal life (Soria-Milla et al., 1987), the first seizure was induced at 30 days of age. Moreover, since it has been demonstrated that AGS susceptibility diminishes with age (Soria-Milla et al., 1987; De Luca et al., 2004), epileptic animals were tested once every 15 days, for a total of 11 tests, under the same conditions. The last seizure was induced 1 month before they were sacrificed at approximately 7—9 months of age. For seizure induction, the auditory stimulus was white noise with a frequency of 1—20 kHz, an intensity of 60—80 dB and duration of 20 s. Control hamsters were stimulated under the same conditions and they never exhibited sound-induced seizures. Hamsters were held under environmentally controlled conditions (06:00 a.m.:20:00 p.m. light/dark cycle, 25 °C, with food and water available ad libitum).

For immunocytochemistry, control ($n = 5$) and epileptic ($n = 5$) adult animals were anesthetized with pentobarbital (60 mg/kg) and perfused with a 0.9% saline wash followed by a fixative solution of 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 (PB). The brains were sectioned at 40 μm on a freezing sliding microtome and processed simultaneously. Coronal sections were incubated overnight at 4 °C in an anti-GABA primary antibody solution (1:1000; Sigma—Aldrich) diluted in TBS (Tris-buffered saline). Then, the tissue was incubated in a 1:200 dilution of anti-rabbit biotinylated secondary antibody (1:200; Vector Laboratories) for 2 h at room temperature. The vector biotin-avidin procedure was used to link the antigen—antibody complex to HRP, which was visualized by using the diaminobenzidine tetrahydrochloride peroxidase histochemistry procedure.

The densitometric analysis method used in this study has been described previously (Alvarado et al., 2004; Fuentes-Santamaría et al., 2005). Briefly, analysis of the immunostaining was performed in every fourth section along the rostrocaudal extent of the IC. Images were captured by using a digital camera, converted to an eight-bit image containing grayscale values from 0 to 255 and normalized. Because densitometric studies have suggested that immunostaining intensity is related to antigen concentration (Huang et al., 1996; Yao and Godfrey, 1997), the mean gray level of the CNIC, the mean gray level of the immunostained neuropil and the mean gray level within neurons were used as indirect indicators of the amount of GABA within the nucleus, neuropil and neurons, respectively. In order to quantify the mean gray level within neurons, three fields (dorsal, middle and ventral) were sampled in each section using a 20× objective, for a total of 12 fields in each animal. Thresh-

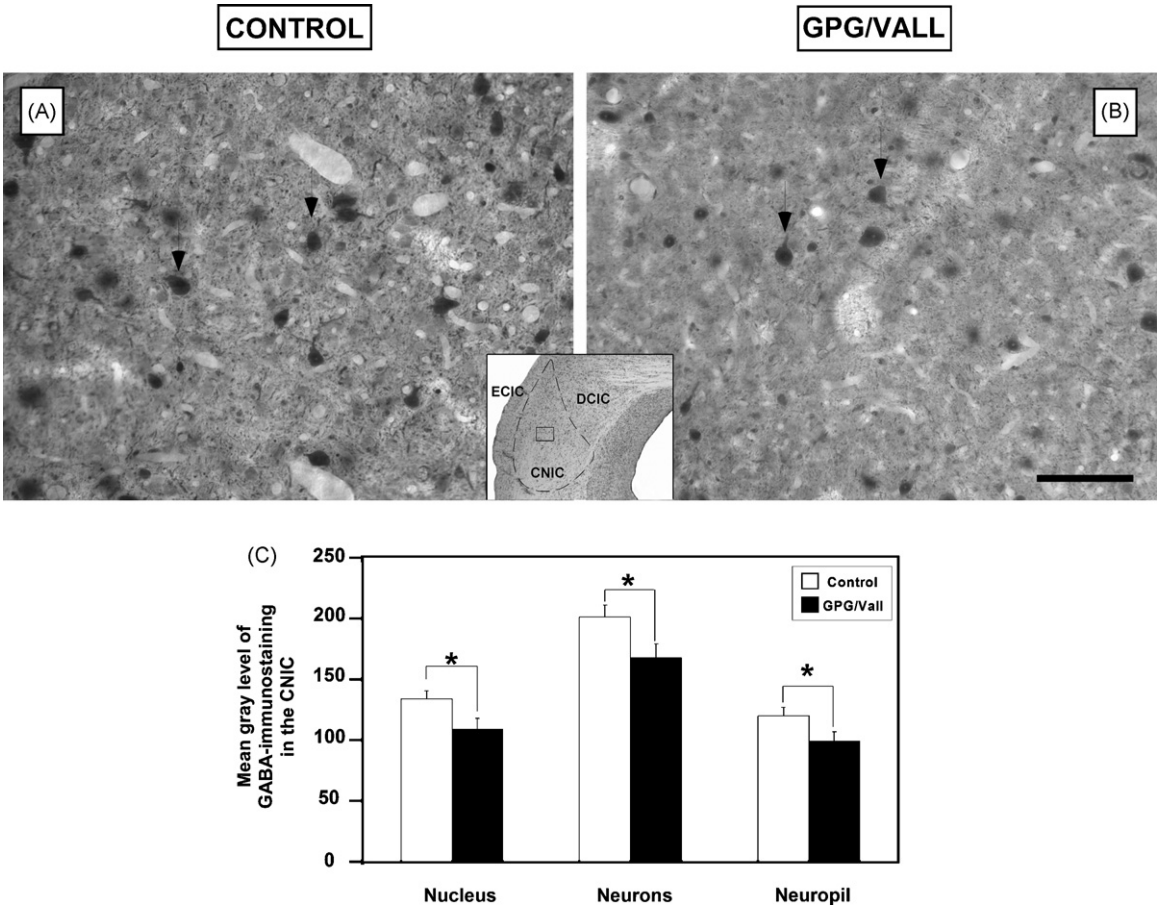

Figure 1 Digital images illustrating GABA immunostaining in the CNIC of control and GPG/Vall hamsters. Note that there is a decrease in GABA immunostaining in the epileptic hamster (B) compared to control (A) animals. Quantification of this decrease indicates that it was due to a decrease both in the mean gray level of the immunostained neuropil and in the mean gray level within immunostained neurons in the epileptic hamster (C). Arrows indicate immunostained neurons. The box in the inset indicates the location of the tissue magnified in A and B. Asterisks (*) in C indicate significant differences between groups. Scale bar for *A*, *B* = 100 μm.

old detection was set as two standard deviations above the value of the field and all the profiles (neurons) exceeding this threshold were identified as labeled and measured. In order to quantify the mean gray level in the neuropil, three fields (dorsal, middle and ventral) containing only neuropil also were sampled in each section using a 40× objective, for a total of 12 fields in each animal (Alvarado et al., 2004). These three measures provided distinct but related information about the changes of GABA in the CNIC.

The HPLC analysis was performed as described elsewhere (Fuentes-Santamaría et al., 2007). Briefly, control ($n = 4$) and epileptic ($n = 4$) animals were decapitated and its head quickly frozen on dry ice. The brain was removed and the IC isolated from the frozen head. Each tissue sample was centrifuged for 20 min and the upper supernatants were frozen at −20 °C for HPLC analysis. Supernatants were neutralized with 0.1N sodium hydroxide and derivatized with ortho-phthal-dialdehyde. The fluorescent derivatized amino acids were separated by a reversed-phase Micra C18 column (33 mm × 4.6 mm, particle size 1.5 mm) using gradient elution. The solvent flow rate was adjusted to 0.5 ml/min and the injection volume was 10 μl. Fluorescence detection (PerkinElmer LS4) was performed at 365 and 455 nm for excitation and emission wavelengths, respectively.

Data were analyzed statistically using a Student's t-test. Statistical significance was determined at a level of $P < 0.05$.

Results

Analysis of sections immunostained for GABA in the CNIC in control hamsters revealed a lightly immunostained neuropil and darkly immunostained neurons that were small in size (arrows in Fig. 1A). The immunostaining within these neurons was observed mainly within the cytoplasm and occasionally in some dendritic processes (Fig. 1A). In comparison with control hamsters, a decrease in GABA immunostaining, reflected as a significant decrease ($t = 4.86$, d.f. = 8, $P < 0.01$) in the mean gray level throughout the CNIC, was observed in the GPG/Vall hamster (compare arrows in Fig. 1B with A; also see C). Further analysis revealed that this decrease was due to a significant decrease both in the mean gray level of the immunostained neuropil ($t = 4.41$, d.f. = 8, $P < 0.01$) and in the mean gray level within neurons ($t = 4.97$, d.f. = 8, $P < 0.01$) in the epileptic animal (Fig. 1C). The rostrocaudal distribution of GABA-immunostained neurons is shown in Fig. 2. Note that there was a qualitative decrease in the number of immunostained neurons in the epileptic animal compared to control hamsters (Fig. 2). HPLC analysis of GABA concentration also revealed a significant decrease in the levels of this amino acid in the CNIC of the GPG/Vall hamster compared to control animals ($t = 5.85$, d.f. = 6, $P < 0.01$; Fig. 3).

Discussion

Evidence suggests that reduction of GABA-mediated inhibition in the CNIC causes seizures in normal animals thus, indicating that GABA modulates seizure susceptibility (Millan et al., 1986). Such a reduction of inhibition has been proposed to result in excessive firing of neurons in the CNIC, and therefore, to an increased predisposition to AGS (Faingold, 2002; Evans et al., 2006). Although the underlying cause of this altered GABAergic function has not been completely elucidated, it has been suggested that it could be due to decreased levels of GABA in the IC (Lasley, 1991). In agreement with this latter study, the current findings also

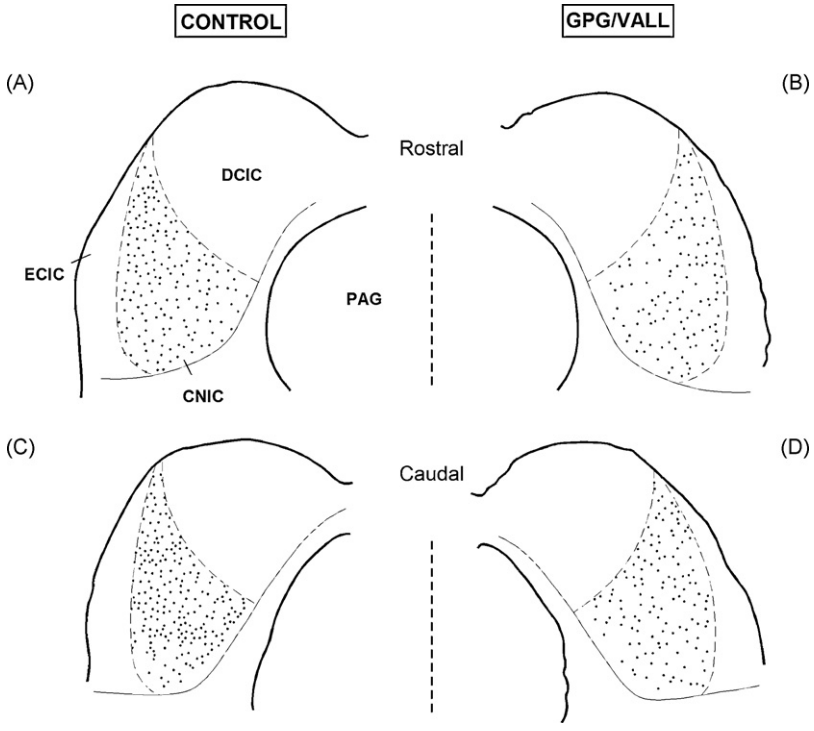

Figure 2 Plots are shown depicting the rostrocaudal distribution of GABA-immunostained neurons in both control (A, C) and epileptic (B, D) animals. Note that there is a qualitative decrease in the number of GABAergic neurons in the epileptic animal compared to control hamsters.

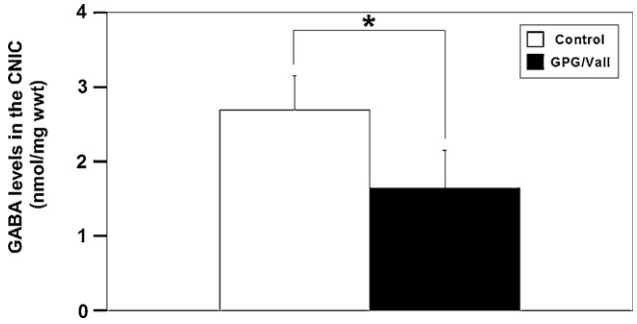

Figure 3 Bar graph showing decreased levels of GABA, as determined by HPLC, in the CNIC in the GPG/Vall hamster compared to control animals. Values are expressed as mean ± S.E. Asterisk (*) indicate significant difference between groups.

report decreased levels of GABA in the CNIC of the epileptic hamster, as determined by densitometry as well as by HPLC analysis. Moreover, the present study demonstrates that this decrease is not only observed within immunostained neurons but also in the immunostained neuropil thus, suggesting a reduced post- and presynaptic availability of this neurotransmitter. In contrast to the decreased levels of GABA reported here in hamsters and also in the genetically epilepsy-prone rat (GEPR-9) (Lasley, 1991), Ribak et al. (1988) observed an increased GABA concentration in the IC in GEPRs-9. Nevertheless, the discrepancy between these last two studies has been proposed to be due, at least partially, to the types of control animals used in each study (Lasley, 1991).

Besides reduced levels of GABA, other factors, including increased expression of GABA transporter, GABA-receptor desensitization and reduced GABA(A)-receptor function after AGS, may account for the abnormalities of GABA-mediated inhibitory neurotransmission in the CNIC (Faingold, 1999; Evans et al., 2006). Altogether, the present data suggest that similar to other animal models of AGS, an abnormal GABAergic system occurs in the CNIC of the GPG/Vall hamster. In addition, they support the findings in other species suggesting an important role for GABA in the mechanism of seizure activity in the CNIC.

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