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Brain Research Protocols 10 (2003) 148–155

BRAIN RESEARCH PROTOCOLS

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Protocol

Immunohistochemistry as a tool for topographical semi-quantification of neurotransmitters in the brain

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Accepted 15 October 2002

Abstract

Immunohistochemistry is a powerful tool to detect neurotransmitter (NT) presence in different brain structures with a high spatial resolution. However, it is only scarcely used in quantitative approach due to lack of reproducibility and sensitivity. We developed a protocol of NT detection based on immunohistochemistry and image analysis to show that this approach could also be useful to evaluate NT content variations. We focused our study on the GABAergic system in the cerebellum and measured different accurate parameters, namely the optical density (O.D.), the stained area and the number of immunoreactive cells in each cerebellar cell layer. In order to modify the GABA content, we used gamma-vinyl-GABA (GVG), an inhibitor of GABA-transaminase, known to dramatically increase GABA concentration in the central nervous system (CNS) and especially in the cerebellum. We observed a significant increase in the three parameters measured in the molecular and the granular layers of the cerebellum after treatment with GVG, reflecting the well-established increase in GABA content after such a treatment. Therefore, our technical approach allows not only a precise determination of the effects in particular cell layers but also a semi-quantification of GABA content variations. This technique could be suitable for monitoring NT variations following any treatment.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: GABA

Keywords: Neurotransmitter; γ-Aminobutyric acid; Immunohistochemistry; Image analysis; Semiquantification; High spatial resolution

The determination of NT concentration variations is of great importance in order to detect the effects of different factors on the CNS homeostasis. The quantification of NT can be accomplished by several different approaches [1,12,20,28] and often requires chromatographic separation by high-performance liquid chromatography (HPLC) [17–19,27]. Although these methods are very useful to determine NT concentrations in tissue homogenates, they do not give any precise information on NT location within a particular brain region. On the contrary, immunohistochemistry (IHC) has been extensively used to precisely locate substances in brain structures [3,6,9,22] but has been only scarcely used as a quantitative method [5,8,23,30]. Image analysis has been frequently used to detect and count cells stained for different markers such as hormones [7,15], proteins [2] or NT in the CNS [3,22,30]. However, few studies used this method to quantify precisely some compound concentrations, for example by using a calibration curve to assign a concentration to an optical density (O.D.) value [11,23].

In the present work, we set up a protocol of IHC and image analysis in order to evaluate cellular γ-aminobutyric acid (GABA) variations in a pharmacological model in which altered GABA concentrations had been measured precisely elsewhere by HPLC [14]. Gamma-vinyl-GABA (GVG) is a selective irreversible inhibitor of GABA-transaminase (GABA-T) [10,24] that induces a dramatic increase in GABA concentration in the CNS [14,21]. The most important effects of GVG have been reported in the

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1385-299X/02/$ – see front matter
PII: S1385-299X(02)00206-4

cerebellum, the hippocampus and the frontal cortex [4,14]. We performed a dose–response study by using increasing doses of GVG and evaluated the presence of GABA by IHC and image analysis in different cell layers. Because the cerebellum is topographically organized in three easily identified cell layers, this structure suited very well to such an approach.

We conclude that IHC coupled with accurate image analysis is a useful method to evaluate modifications in NT quantities in defined brain structures and even in cell layers.

1. Type of research

This protocol can be used in any type of research that requires the semi-quantitative determination of NT content in precise brain structures. Moreover, the protocol was designed for GABAergic neurons but it could also be used for other types of neurons as well as for other cells present in the CNS (like glial cells) or in any tissue.

- Application of immunohistochemistry coupled with image analysis to semi-quantitative neuroscientific studies
- Determination of GABA quantity variations after pharmacological treatment

2. Time required

- The effect of the i.p. GVG is maximum after 4 h
- Tissue fixation requires about 5 min after deep anesthesia of the animal
- Dissection of cerebellum from rat brain requires about 5 min following decapitation
- The time required for the whole IHC experiment is about 2 days:
  - Incubation with the primary antibody: 48 h
  - Incubation with the secondary antibody: 1 h
  - Incubation with the tertiary antibody: 1 h
  - All washing steps: 1 h
  - Incubation with the revelation system (DAB): 8 min
- The section mounting on glass slides requires about 5 min per cerebellum (six sections), and the drying of slides requires 24 h. After coverslipping with mounting medium, the drying requires 3 additional days
- Image analysis of six slices obtained from one cerebellum (one rat) requires about 10 min

3. Materials

3.1. Equipment

- Peristaltic pump (any supplier)
- Vibratome (any supplier)
- Upright optical microscope (Leica DMLB) coupled with a Color Camera Tri CCD (Donpisha) and image analysis software IPS 4.06 (UNILOG Alcatel, Grenoble, France)

3.2. Chemicals and reagents

- Drug: Gamma-vinyl-GABA (GVG) was generously supplied by Marion Merrell Bourgoin S.A. (Bourgoin-Jallieu, France)
- Antibodies: the rabbit GABA-polyclonal antibody was a gift from M. Geffard (Bordeaux, France), the goat anti-rabbit immunoglobulin G was purchased from Tebu (Le-Perray-en-Yvelines, France) and the rabbit peroxidase–antiperoxidase complex was purchased from DAKO S.A. (Trappes, France)
- Trypsin–EDTA was purchased from Gibco Invitrogen (Cergy Pontoise, France)
- 3,3′-Diaminobenzidine hydrochloride and all other chemicals were of analytical grade and were purchased from Sigma–Aldrich (Strasbourg, France)

3.3. Animals

Male Wistar rats (6 weeks, body weight about 180 g) were purchased from Iffa-Credo (France). The rats were accommodated to the laboratory 1 week before the beginning of the experiment, kept under controlled environmental conditions (ambient temperature 22 °C, 12:12 h light/dark cycle) and received food and tap water ad libitum. All the animals weighed 220–250 g on the day of the experiment. Animals were cared for and surgically handled in accordance with the European Communities Council Directive (24 November 1986, 86/609/EEC).

4. Detailed procedure

4.1. Drug treatment

GVG was administered i.p. at different dosages: 1, 100 and 1200 mg/kg. Control rats were injected with 0.9% saline. Groups of six rats per dose were used in this experiment. All drugs were freshly dissolved and administered at a volume of 3 ml/kg.

4.2. Dissection of the brains

The animals were deeply anaesthetized with sodium pentobarbital 6% (i.p.) 4 h after the injection of GVG. Tissue fixation was achieved by a transcardially perfusion of 15 ml cacodylate 50 mM/sodium bisulfite 1% buffer (pH 7.6) followed by 500 ml of 5% glutaraldehyde (in the same buffer) delivered by a peristaltic pump (Masterflex) at 200 ml/min for 1 min followed by a lower rate at 100

ml/min for 3 min. The brains were removed from the skull and postfixed in the same fixative at 4 °C until the IHC experiment.

4.3. Immunohistochemistry

Cerebellum was separated from the rest of the brain. Sagittal sections were cut with a Vibratome in order to avoid dehydration or freezing steps which might alter the tissue. Sections of 50 μm thickness were used to preserve as much as possible the tissue structure during free-floating processes. Cerebellar sections were then stained for GABA immunodetection. They were pretreated with trypsin 0.05%–EDTA 0.02% (5 min, Gibco). After washing (2×5 min) in Tris Buffer Saline (TBS: Tris 0.05 M, NaCl 9‰, pH 7.6), sections were incubated with a rabbit polyclonal antibody against GABA (Geffard, France) diluted at 1:5,000 in TBS containing goat non specific serum (NSS) 1% and Triton 0.1% for 48 h at 4 °C. After washing in TBS (2×10 min), sections were incubated with a goat anti-rabbit immunoglobulin G (Tebu) diluted at 1:200 in TBS (containing goat NSS 1%) for 1 h at room temperature, washed 2×5 min in TBS and incubated with a rabbit peroxidase–antiperoxidase complex (DAKO) diluted at 1:200 in TBS (containing goat NSS 1%) for 1 h at room temperature. After the last washes (2×5 min) in TBS, immunoreactivity was revealed with 0.04% 3,3′-diaminobenzidine hydrochloride (DAB, Sigma) diluted in TBS (pH 7.6) in the presence of 0.02% H_2O_2. Particular care was taken to ensure a comparable staining procedure between control and experimental cerebellar sections: (i) the duration of the incubation in the DAB-H_2O_2 solution was strictly controlled and fixed at 8 min for all the sections, (ii) the control and experimental cerebella were always processed in parallel. The reaction was terminated by rinsing several times in TBS. The sections were then mounted on glass slides in 0.06 M phosphate buffer, dried for 24 h at ambient temperature and coverslipped with DePeX mounting medium (Gurr). They were dried for at least 3 days before examination under the microscope and further image analysis.

4.4. Image analysis

An upright optical microscope (Leica DMLB) coupled with a Color Camera Tri CCD (Donpisha) and image analysis software IPS 4.06 (UNILOG Alcatel) were used. To highlight the brown peroxidase staining, a neutral filter (N4) was inserted. The software was improved (in collaboration with Unilog, Grenoble, France) and adapted to our aim, which was to measure parameters reflecting the GABA quantity in the stained cells. Three parameters were chosen for their reliability with GABA concentration in neuronal cells: (i) the optical density (O.D.) of immunostained cells (staining intensity of the cells is proportional to the local GABA concentration), (ii) the percentage of stained profiles area in the different analyzed fields (the stained profile reflects the NT extent in the cell layers) and (iii) the number of stained cells in these analyzed fields (this last parameter reflects only huge variations in NT quantities). By means of CCD camera, O.D. values were calculated by common logarithmic transform of the ratio of incident to transmitted light. The whole O.D. range (0 to +2) was divided into 256 digitized values (0–255, corresponding to 8 bits) to highlight variations in staining intensity. All the parameters described above were measured for each cell detected as positive, i.e. with a minimal size (5 μm) and an O.D. value higher than a defined threshold. This threshold was chosen as the O.D. value above which only cell bodies (and not processes) were detectable in the molecular and granular layers. It was therefore very important to readjust the threshold value for each section to obtain the optimal conditions to detect only the cell bodies and discard some artifacts that would induce a misevaluation of the parameters. Interestingly, in the Purkinje cell layer, the accumulation of GABA in basket terminals was also detected with these parameters.

The analysis was performed on live acquired images of 768×576 pixels (corresponding to a tissue surface of 0.043 mm^2, objective 20, ocular 2.5) in each cerebellar cell layer (molecular, Purkinje cell and granular layers) of control and GVG-treated animals. Six sections per rat and three fields per section (corresponding to the three cell layers in the cerebellum) were analyzed. Each analyzed field was chosen randomly in each cerebellar cell layer of each section and delimited by the experimenter through interaction with the software. The mean O.D. (M.O.D.) value was obtained by averaging O.D. values of all stained profiles in this field. The O.D. of the glass slide, as well as the background O.D. of each section (measured by the software all around the positive cells) was systematically subtracted from this M.O.D. A single M.O.D. value was then obtained for each layer of each section. As the analysis was carried out on six sections per rat, six M.O.D. values were obtained for each cell layer in each animal. The percentage of the stained area in the analyzed field was calculated as the ratio between the stained area and the total area of the analyzed field in one layer. Thus, six values of the percentage of stained area were obtained for each cell layer in each rat. The number of stained cells was obtained by counting individual positive cell bodies in each layer of each analyzed field. Six values of stained cell number were then calculated per cell layer and per rat. In the Purkinje cell layer, this number was not calculated as we detected only the basket terminals there.

4.5. Statistical analysis

A Mann–Whitney test was used to compare the effects of control and GVG treatments on each parameter. The

comparisons were performed on 36 values per group (six sections per rat, six rats per group).

Differences were considered significant when $P<0.05$ ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

5. Results

As stated above, the M.O.D. value reflects the local GABA concentration in the cells. In the molecular layer, we noticed a significant increase in the M.O.D. value after treatment with the highest doses of GVG (100 and 1200 mg/kg) compared to control (34% and 32% increase, respectively, $P<0.01$, Fig. 1) and GVG 1 mg/kg (27% and 25% increase, respectively, $P<0.05$, Fig. 1). In the Purkinje cell layer and the granular layer, no statistical significant difference was observed, even if a tendency to a slight increase in the M.O.D. could be noticed after treatment with GVG 100 and 1200 mg/kg compared to control (Fig. 1).

The area of immunoreactive profiles indicates the extent of GABA in the cell layer. In the molecular layer, this parameter was significantly increased after treatment with GVG 1200 mg/kg compared to control (37% of increase, $P<0.001$), GVG 1 mg/kg (56% of increase, $P<0.001$) and 100 mg/kg groups (26% of increase, $P<0.01$, Fig. 2). In the granular layer, we observed a significant increase in this parameter after treatment with GVG 1200 mg/kg as well (30% of increase compared to control group, $P<0.05$ and 41% of increase compared to GVG 1 mg/kg group, $P<0.01$, Fig. 2). In the Purkinje cell layer, no statistically significant difference was observed between treated and control groups (Fig. 2).

The last parameter measured was the number of immunoreactive cell bodies which is affected only when the GABA content of the cells varies dramatically (see Section 4). This number was highly affected by GVG treatment both in the molecular and granular layers. In the former, we noticed a significant increase in the number of immunoreactive cells after treatment with GVG 1, 100 and 1200 mg/kg compared to controls (20%, 23% ($P<0.05$) and 48% ($P<0.001$) increase, respectively, Fig. 3). We also observed a significant increase after treatment with the highest dose of GVG (1200 mg/kg) compared to treatment with lower doses 1 and 100 mg/kg (23% and 20% increase, respectively, $P<0.01$). In the granular layer, we obtained a significant increase after treatment with GVG 1200 mg/kg compared to controls (52% increase, $P<0.001$) and 1 mg/kg (27% increase, $P<0.05$). The number of immunoreactive cells was also increased after treatment with GVG 100 mg/kg compared to control (47% increase, $P<0.001$, Fig. 3).

Fig. 4 illustrates the difference of staining between cerebellar sections of control, GVG 1, 100 and 1200 mg/kg treated rats. Note the difference of staining of the cell bodies between control and treated animals in the molecular and granular layers (Fig. 4A–D). The difference of staining between control and GVG 1200 mg/kg is highlighted when observed at higher magnification (Fig. 4E,F).

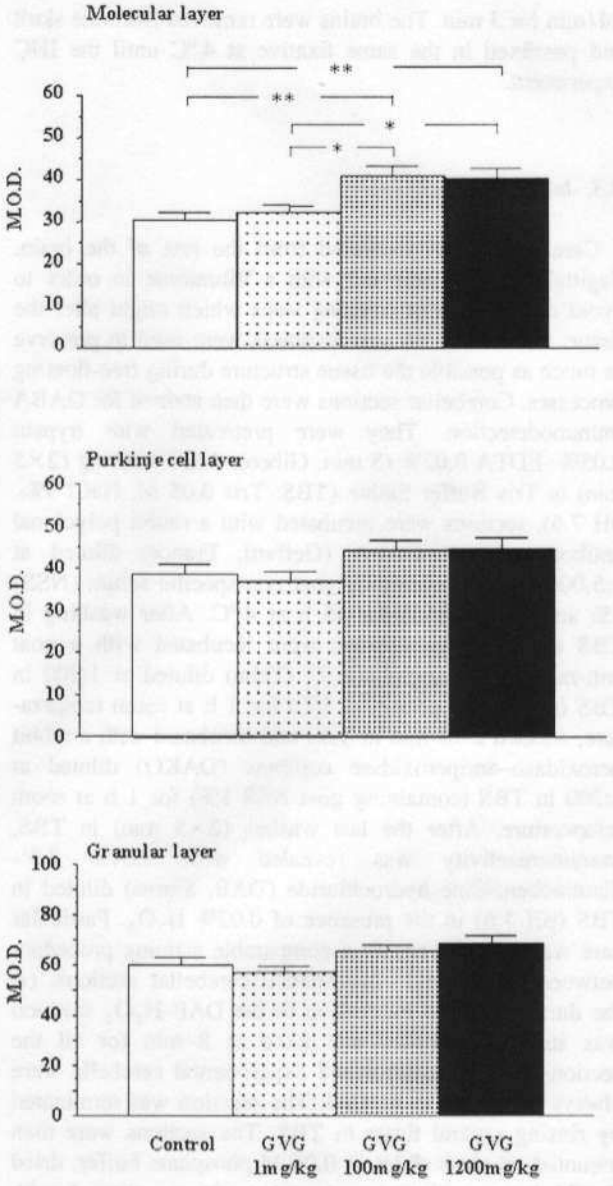

Fig. 1. Effect of GVG on the mean optical density (M.O.D.) of GABAergic cells in the rat cerebellum. Each column represents the mean ± S.E.M. of 36 values (six rats, six values per rat). $^{*}P<0.05$, $^{**}P<0.01$.

To summarize, our technical approach allowed to establish the well-known increase in GABA content induced by GVG treatment and to distinguish this effect in the different cell layers of the cerebellum.

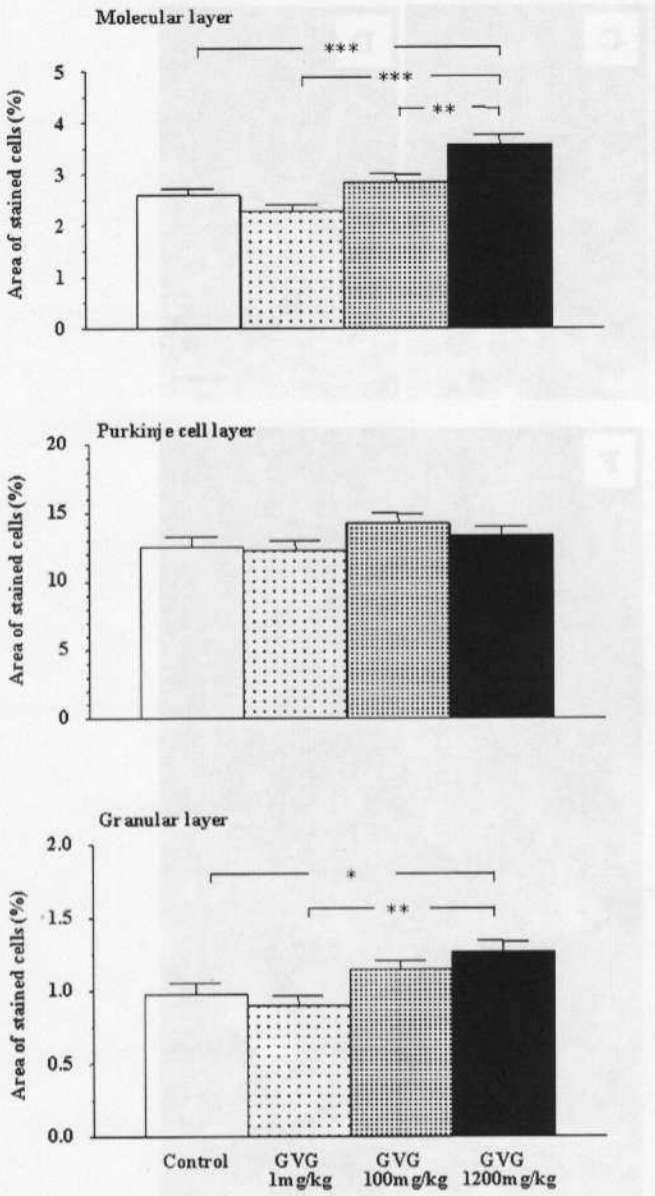

Fig. 2. Effect of GVG on the percentage of GABA immunoreactive area in the rat cerebellum. Each column represents the mean±S.E.M. of 36 values (six rats, six values per rat). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

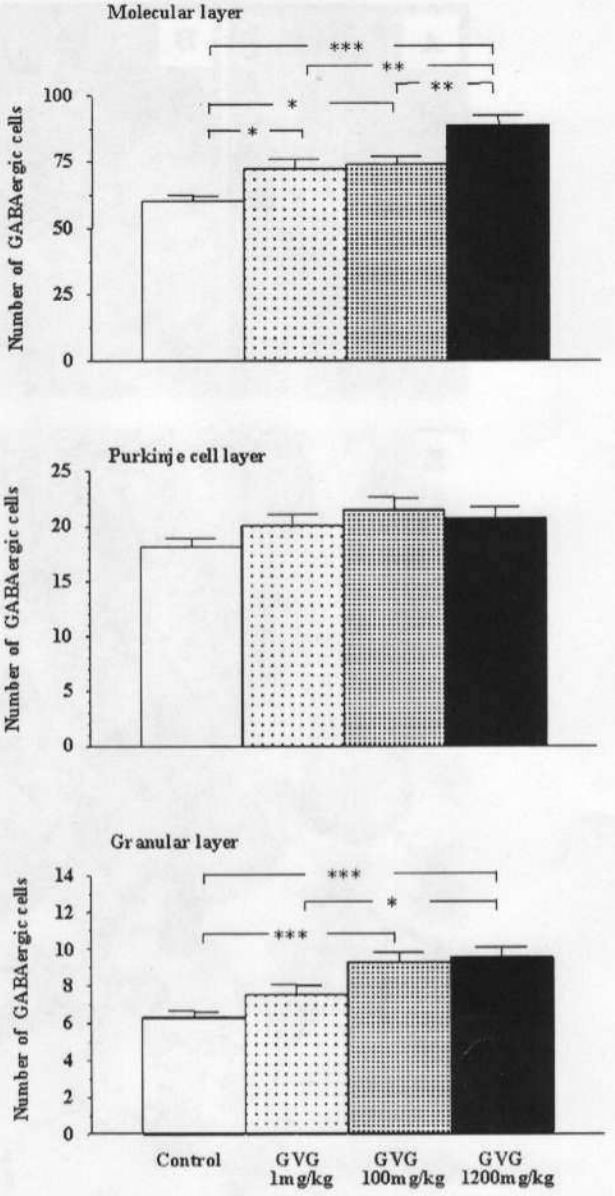

Fig. 3. Effect of GVG on the number of GABAergic immunoreactive cells in the rat cerebellum. Each column represents the mean±S.E.M. of 36 values (six rats, six values per rat). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

6. Discussion

In this study, we showed that IHC allows the detection of variations in the cellular GABA content when coupled with appropriate image analysis. We chose to use this method (rather than HPLC) to obtain specific information on the location of NT concentration variations inside a particular brain structure and, more precisely, in different cell layers. Actually, quantitative approaches such as HPLC or capillary electrophoresis constitute appropriate methods to measure precisely NT concentration values [19,20] in order to obtain, for instance, kinetic curves and could therefore be used as fully complementary methods of our technique. However, since subtle differences concerning one specific cell or a given circuit can be overlooked by these techniques, IHC coupled with image analysis seems to be the method of choice to get deeper insights into the local variations in NT quantities.

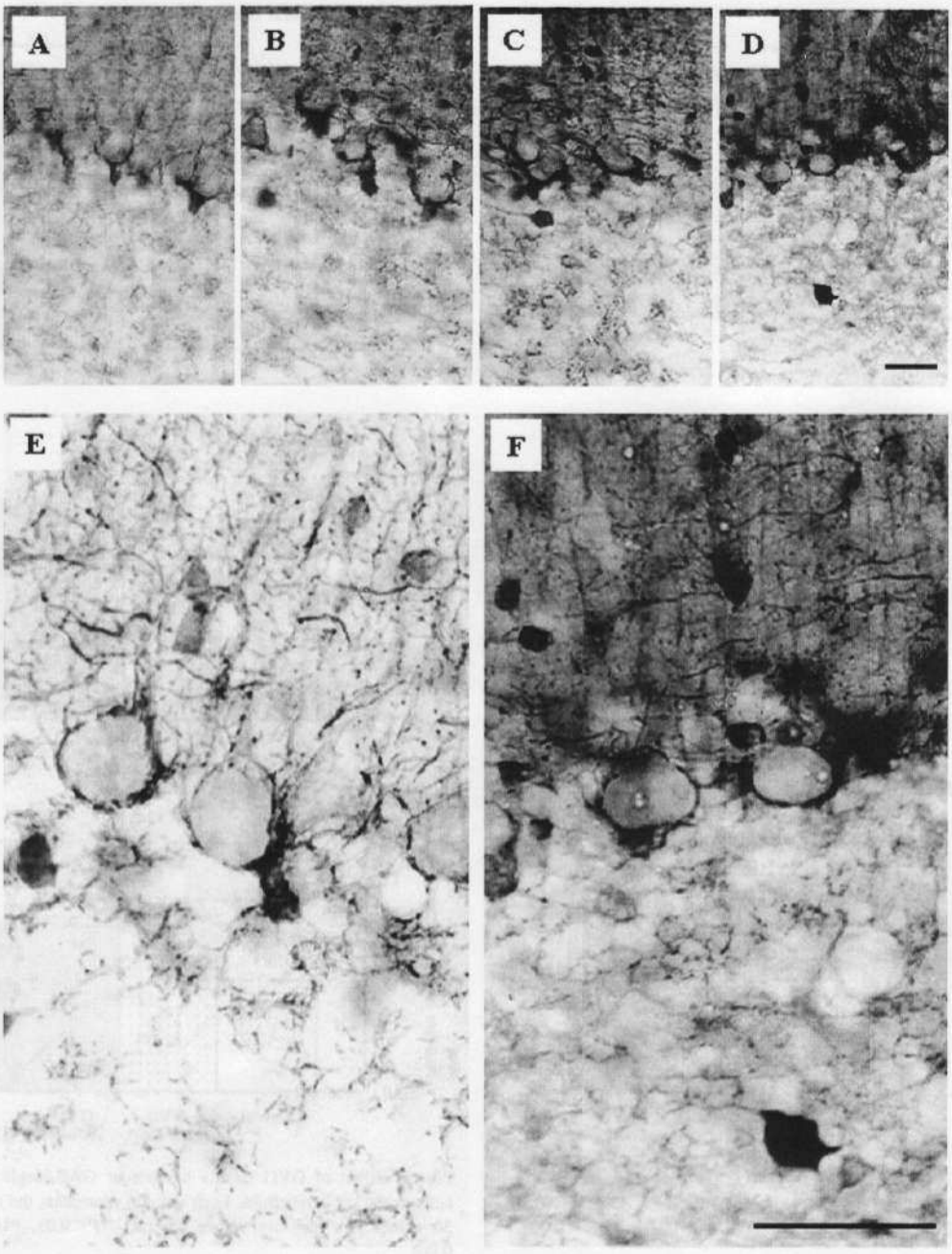

Fig. 4. Effect of GVG treatment on the staining of GABAergic cells in the rat cerebellum. Each photograph contains the molecular layer (upper part), the Purkinje cell layer (middle part) and the granular layer (lower part). (A–D) Views at low magnification (×250): (A) control, (B) GVG 1 mg/kg, (C) GVG 100 mg/kg, (D) GVG 1200 mg/kg. (E,F) Views at higher magnification (×500): (E) control, (F) GVG 1200 mg/kg. Scale bars=50 μm.

First of all, we had to develop a reproducible IHC protocol and consequently to define different accurate parameters to reflect the intracellular GABA content in the immunoreactive cells. The reproducibility of IHC was achieved by treating control and experimental sections in parallel and by strictly controlling the duration of the staining reaction (DAB incubation). The different parameters measured were chosen in order to give different and complementary information about the cellular GABA content. Indeed, the O.D. values reflected the local GABA concentration inside each cell [23], the stained area indicated rather the extent of GABA in the cell layer, and the number of stained cell bodies displayed the amplitude of GABA content changes in the cells.

The determination of these parameters required the definition of an appropriate threshold for cell detection.

This threshold, defined as the O.D. value above which cell bodies can be detected in their totality, had to be reset for each section since the staining of the cells could vary between the sections. A special care was given to this step and the stained areas corresponding to unidentified 'phantoms' were systematically discarded from the analysis. To obtain comparable data between sections, it was also necessary to subtract the background O.D. of each section and of each glass slide from the M.O.D. of the cells.

We were able to distinguish the effect of GVG in the three different cell layers of the cerebellum with a good cellular resolution. In the Purkinje cell layer, no obvious changes were observed. This could be explained by the fact that GVG acts essentially in the perikarya and less so in the processes [26]. Therefore, the GABA content of basket terminals detected in the Purkinje cell layer was not affected by this treatment. On the contrary, all the three parameters measured (M.O.D., area and number of stained cells) were increased both in the molecular and the granular layers of GVG treated animals. This increase was much more pronounced with the highest doses of GVG (mainly in the molecular layer).

Several HPLC studies have related the effects of GVG on the brain GABA content and concluded that GVG induces a huge increase in GABA concentration [14,24]. For instance, Löscher and Höstermann [14] showed that GVG (1200 mg/kg) induced a 300% increase in the GABA content in the cerebellum. With our technical approach, we were able to find increases in intracellular GABA content reaching 150% of the control values. We could explain the difference between our results and those of Löscher and Hörstermann [14] by (1) the difference of the cellular compartment measured: the intracellular one in our investigation vs. both intra- and extracellular in their work, and (2) the type of cells measured: neuronal cells in our study vs. both neuronal and glial cells in theirs. Indeed, it was shown that GVG was able to increase the GABA content in nerve terminals [13,25] but GABA-T is mainly localized in non-synaptosomal mitochondria, especially in glial cells [21,26,29]. Therefore, the effect of GVG measured on neuronal cells would constitute a small part of the global effect of GVG. We did not evaluate the GABA content in glial cells since they were too small to be taken into account.

As a conclusion, our technical approach is suitable for monitoring the effects of various factors on NT concentrations in the CNS and detecting these effects with a high spatial resolution. In this regard, it has recently been successfully used to detect the effects induced by an in vivo exposure to electromagnetic fields emitted by mobile phones on the cerebellar GABA concentration in rats [16].

7. Trouble shooting

1. The duration of incubation with DAB during IHC process must be strictly controlled to minimize staining variations between sections.
2. The detection of positive cells depends on the threshold fixed by the experimenter. In order to avoid bias induced by this step, different independent experimenters should perform this analysis.
3. To limit the variability of the measures, it is necessary to calculate the parameters on several sections per animal.

8. Quick procedure

1. Injection of GVG at different dosages (1, 100 and 1200 mg/kg)
2. Tissue fixation and dissection of the brains and cerebelli
3. IHC experiment and section mounting on microscope slides
4. Image analysis
5. Data analysis

9. Essential literature references

[16,23]

Acknowledgements

We thank Jean-René Teilhac for his excellent artwork and Françoise Montpeyroux for help in statistical analysis. This work was funded by France Telecom R&D (project 98 1B 025) and the French Ministry of Research (COMOBIO project 99.S.0168).

References

[1] J. Bergquist, M.J. Vona, C.-O. Stiller, W.T. O'Connor, T. Falkenberg, R. Ekman, Capillary electrophoresis with laser-induced fluorescence detection: a sensitive method for monitoring extracellular concentrations of amino acids in the periaqueductal grey matter, J. Neurosci. Methods 65 (1996) 33–42.

[2] J.G. Bilsland, S.J. Harper, Quantification of Fos immunoreactivity in cortical cultures treated with growth factors, J. Neurosci. Methods 84 (1998) 121–130.

[3] R. Buffa, O. Crivelli, C. Lavarini, F. Sessa, G. Verme, E. Solcia, Immunohistochemistry of brain 5-hydroxytryptamine, Histochemistry 68 (1980) 9–15.

[4] A.G. Chapman, K. Riley, M.C. Evans, B.S. Meldrum, Acute effects of sodium valproate and γ-vinyl GABA on regional amino acid metabolism in the rat brain: incorporation of 2-[^{14}C]glucose into amino acids, Neurochem. Res. 7 (1982) 1089–1105.

[5] J.R. Clements, A.J. Beitz, T.F. Fletcher, M.A. Mullett, Immunocytochemical localization of serotonin in the rat periaqueductal gray: a quantitative light and electron microscopy study, J. Comp. Neurol. 236 (1985) 60–70.

[6] A.C. Cuello, G. Galfre, C. Milstein, Detection of substance P in the

central nervous system by a monoclonal antibody, Proc. Natl. Acad. Sci. USA 76 (1979) 3532–3536.
[7] M. El-Sahly, O. Sandström, E. Nässtrom, M. Mustajbasic, S. Zachrisson, Application of computer image analysis in endocrine cell quantification, Histochem. J. 29 (1997) 249–256.
[8] D.S. Gross, J.M. Rothfeld, Quantitative immunocytochemistry of hypothalamic and pituitary hormones: validation of an automated, computerized image analysis system, J. Histochem. Cytochem. 33 (1985) 11–20.
[9] L. Jojich, R.G. Pourcho, Glutamate immunoreactivity in the cat retina: a quantitative study, Vis. Neurosci. 13 (1996) 117–133.
[10] M.J. Jung, B. Lippert, B.W. Metcalf, P. Böhlen, P.J. Schechter, γ-vinyl GABA (4-amino-hex-5-enoic acid), a new selective irreversible inhibitor of GABA-T: effects on brain GABA metabolism in mice, J. Neurochem. 29 (1977) 797–802.
[11] A. Kastner, E.C. Hirsch, M.T. Herrero, F. Javoy-Agid, Y. Agid, Immunocytochemical quantification of tyrosine hydroxylase at a cellular level in the mesencephalon of control subjects and patients with Parkinson's and Alzheimer's disease, J. Neurochem. 61 (1993) 1024–1034.
[12] N. Lindefors, E. Brodin, U. Ungerstedt, Microdialysis combined with a sensitive radioimmunoassay: a technique for studying in vivo release of neuropeptides, J. Pharmacol. Methods 17 (1987) 305–312.
[13] W. Löscher, Effect of inhibitors of GABA aminotransferase on the metabolism of GABA in brain tissue and synaptosomal fractions, J. Neurochem. 36 (1981) 1521–1527.
[14] W. Löscher, D. Hörstermann, Differential effects of vigabatrin, γ-acetylenic GABA, aminooxyacetic acid, and valproate on levels of various amino acids in rat brain regions and plasma, Naunyn-Schmiedeberg's Arch. Pharmacol. 349 (1994) 270–278.
[15] T. Matsuo, H. Mori, Y. Nishimura, T. Maeda, J.-I. Nakagawa, A. Obashi, Quantification of immunohistochemistry using an image analyser: correlation with hormone concentrations in pituitary adenomas, Histochem. J. 27 (1995) 989–996.
[16] A.L. Mausset, R. de Sèze, F. Montpeyroux, A. Privat, Effects of radiofrequency exposure on the GABAergic system in the rat cerebellum: clues from semi-quantitative immunohistochemistry, Brain Res. 912 (2001) 33–46.
[17] S. Murai, H. Saito, E. Abe, Y. Masuda, T. Itoh, A rapid assay for neurotransmitters amino acids, aspartate, glutamate, glycine, taurine, γ-aminobutyric acid in the brain by high-performance liquid chromatography with electrochemical detection, J. Neural Transm. Gen. Sect. 87 (1992) 145–153.
[18] M.F. Olive, K.K. Mehmert, C.W. Hodge, Microdialysis in the mouse nucleus accumbens: a method for detection of monoamine and amino acid neurotransmitters with simultaneous assessment of locomotor activity, Brain Res. Protocols 5 (2000) 16–24.
[19] J. Opacka-Juffry, F. Tacconelli, C.W. Coen, Sensitive method for determination of picogram amounts of epinephrine and other catecholamines in microdissected samples of rat brain using liquid chromatography with electrochemical detection, J. Chromatogr. 433 (1988) 41–51.
[20] O. Orwar, K. Jardemark, I. Jacobson, A. Moscho, H.A. Fishman, R.H. Scheller, R.N. Zare, Patch-clamp detection of neurotransmitters in capillary electrophoresis, Science 272 (1996) 1779–1782.
[21] R.E. Paulsen, F. Fonnum, Regulation of transmitter γ-aminobutyric acid (GABA) synthesis and metabolism illustrated by the effect of γ-vinyl GABA and hypoglycemia, J. Neurochem. 50 (1988) 1151–1157.
[22] G. Pelletier, Identification of endings containing dopamine and vasopressin in the rat posterior pituitary by a combination of radioautography and immunocytochemistry at the ultrastructural level, J. Histochem. Cytochem. 31 (1983) 562–564.
[23] R. Peretti-Renucci, C. Feuerstein, M. Manier, P. Lorimier, M. Savasta, J. Thibault, N. Mons, M. Geffard, Quantitative image analysis with densitometry for immunohistochemistry and autoradiography of receptor binding sites. Methodological considerations, J. Neurosci. Res. 28 (1991) 583–600.
[24] M. Qume, P.S. Whitton, L.J. Fowler, The effect of chronic treatment with the GABA transaminase inhibitors γ-vinyl-GABA and ethanolamine-*O*-sulphate on the in vivo release of GABA from rat hippocampus, J. Neurochem. 64 (1995) 2256–2261.
[25] S. Sahran, N. Seiler, Metabolic inhibitors and subcellular distribution of GABA, J. Neurosci. Res. 4 (1979) 399–421.
[26] L. Salganicoff, E. De Robertis, Subcellular distribution of the enzymes of the glutamic acid, glutamine and γ-aminobutyric acid cycles in rat brain, J. Neurochem. 12 (1965) 287–309.
[27] S. Sarre, Y. Michotte, P. Herregodts, D. Deleu, N. De Klippel, G. Ebinger, High-performance liquid chromatography with electrochemical detection for the determination of levodopa, catecholamines and their metabolites in rat brain dialysates, J. Chromatogr. 575 (1992) 207–212.
[28] H.G. Schulze, L.S. Greek, C.J. Barbosa, M.W. Blades, B.B. Gorzalka, R.F.B. Turner, Measurement of some small-molecule and peptide neurotransmitters in-vitro using a fiber-optic probe with pulsed ultraviolet resonance Raman spectroscopy, J. Neurosci. Methods 92 (1999) 15–24.
[29] A. Sellström, L.B. Sjöberg, A. Hamberger, Neuronal and glial systems for γ-aminobutyric acid metabolism, J. Neurochem. 25 (1975) 393–398.
[30] J. Vilaplana, M. Lavialle, A method to quantify glial fibrillary acidic protein immunoreactivity on the suprachiasmatic nucleus, J. Neurosci. Methods 88 (1999) 181–187.