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# GAMMA-HYDROXYBUTYRATE, ACTING THROUGH AN ANTI-APOPTOTIC MECHANISM, PROTECTS NATIVE AND AMYLOID-PRECURSOR-PROTEIN-TRANSFECTED NEUROBLASTOMA CELLS AGAINST OXIDATIVE STRESS-INDUCED DEATH

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**Abstract—Clinical observations suggested that gamma-hydroxybutyrate (GHB) protects nerve cells against death but the direct proofs are missing. Here, we combined several approaches to investigate GHB capacity to protect human neuroblastoma SH-SY5Y cells against hydrogen peroxide ($H_2O_2$)-induced death. To increase the patho-physiological relevancy of our study, we used native SH-SY5Y cells and SH-SY5Y cells stably transfected with the wild-type amyloid-precursor-protein (APPwt) or control-vector-pCEP4. Trypan Blue exclusion and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide) assays combined with pharmacological analyses showed that $H_2O_2$ reduced native and genetically modified cell viability and APPwt-transfected cells were the most vulnerable. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and activated caspase-3 staining assessed by flow cytometry revealed a basally elevated apoptotic signal in APPwt-transfected cells. Reverse-transcription, real-time quantitative polymerase chain reaction (qPCR) and Western blotting showed that mRNA and protein basal ratios of apoptotic modulators Bax/Bcl-2 were also high in APPwt-transfected cells. GHB efficiently and dose-dependently rescued native and genetically modified cells from $H_2O_2$-induced death. Interestingly, GHB, which strongly decreased elevated basal levels of TUNEL-staining, activated caspase 3-labeling and Bax/Bcl-2 in APPwt-transfected cells, also counteracted $H_2O_2$-evoked increased apoptotic markers in native and genetically modified SH-SY5Y cells. Since GHB did not promote cell proliferation, anti-apoptotic action through the down-regulation of Bax/Bcl-2 ratios and/or caspase 3 activity appears as a critical mechanism involved in GHB-induced protection of SH-SY5Y cells against APPwt-overexpression- or $H_2O_2$-evoked death. Altogether, these results, providing multi-parametric evidence for the existence of neuroprotective action of GHB, also open interesting perspectives for the development of GHB analog-based strategies against neurodegeneration or nerve cell death.**


**Key words: anti-apoptotic drug, anti-oxidant drug, mechanisms of action of GHB, neuroprotection, Alzheimer's disease.**

## INTRODUCTION

Synthetic or exogenous gamma-hydroxybutyrate (GHB) is used since several years in human clinic for various approved indications, including narcolepsy, cataplexia, anesthesia, sleep induction and alcohol withdrawal treatment. During the 3 last decades, various studies demonstrated that, in fact, the mammalian brain is capable of synthesizing endogenous GHB which acts as a neuromodulator possessing most properties of neurotransmitters (Maitre, 1997; Maitre et al., 2000; Crunelli et al., 2006; Andriamampandry et al., 2007; Carter et al., 2009; Coune et al., 2010; Kemmel et al., 2010). It is well admitted that micromolar concentrations of endogenous GHB may be directly derived from GABA metabolism, particularly from the successive transamination and reduction of GABA in the neuronal compartment by GABA-transaminase and aldo-keto-reductase (Lyon et al., 2007). Endogenous GHB may reduce GABA release *in vivo* and exert a feed-back inhibition on the activity of GABA synapses via GHB

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*Abbreviations:* AD, Alzheimer's disease; APPwt, amyloid-precursor-protein; BSA, bovine serum albumin; DMEM, Dubelcco's modified eagle medium; EDTA, ethylenediaminetetraacetic acid; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GHB, gamma-hydroxybutyrate; $H_2O_2$, hydrogen peroxide; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide; NADPH, nicotinamide adenine dinucleotide phosphate; OD, optical density; PBS, phosphate-buffered saline; PE, phycoerythrin; qPCR, quantitative polymerase chain reaction; SDS, sodium dodecyl sulfate; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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receptors that have also been characterized in the rodent and human brain (Hu et al., 2000; Maitre et al., 2000; Andriamampandry et al., 2003, 2007; Coune et al., 2010). Clinical observations of head injury-induced coma in humans and various investigations in animal models of cerebral ischemia or hypoxia revealed that GHB treatment significantly reduced histological and functional damages evoked by transient global lesion, four-vessel occlusion or kainic acid-induced brain impairment (Wolfson et al., 1977; Vergoni et al., 2000; Ottani et al., 2003). Altogether, these data strongly suggest that GHB may efficiently protect nerve cells against apoptosis or death mechanisms. However, *in vitro* experiments showing cellular, molecular and pharmacological evidence supporting GHB-induced neuroprotective effects have never been performed. The present study was therefore undertaken to check whether or not GHB may exert a beneficial effect on nerve cell survival and protect them efficiently against hydrogen peroxide ($H_2O_2$)-induced oxidative stress and cell death. Indeed, oxidative stress, generated by excessive amounts of reactive oxygen species (ROS) including $H_2O_2$, is a major pathogenic process identified as a mediator of brain damages caused by the accumulation of β-amyloid peptides (Aβ) in Alzheimer's disease (AD) patients (Behl et al., 1994; Beal, 1995; Mizuno et al., 1998; Yatin et al., 1999; Miranda et al., 2000; Giasson et al., 2002; Tamagno et al., 2003; Andersen, 2004; Tabner et al., 2005; Citron, 2010; Oda et al., 2010; Ballard et al., 2011). To provide valuable proofs and physiologically relevant evidence for the existence of GHB protective effect against cell death, we combined several approaches to investigate the ability of GHB to counteract $H_2O_2$-induced death of native and genetically modified human neuroblastoma SH-SY5Y cells produced by the stable transfection of DNA constructs harboring human wild-type amyloid-precursor-protein (APPwt) or the control expression vector pCEP4 (Scheuermann et al., 2001). In a first step, we coupled Trypan Blue exclusion analysis with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium-bromide (MTT) reduction assays to ensure an accurate determination of SH-SY5Y cell survival and viability. Afterward, we used the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) and activated caspase-3 staining assessed by flow cytometry to determine the level of apoptotic signal in native, APPwt- and control vector-transfected SH-SY5Y cells in the absence (basal conditions) or presence of $H_2O_2$. Furthermore, we combined reverse transcription, real-time quantitative polymerase chain reaction (qPCR) and Western blot analyzes to investigate mRNA and protein levels of key pro- or anti-apoptotic factors, including Bax and Bcl-2 (Eckert et al., 2003a,b; Jung et al., 2009; Zhang et al., 2009; Zarate et al., 2010), in native and genetically modified SH-SY5Y cells under normal or $H_2O_2$-induced oxidative stress conditions. Pharmacological studies were performed to assess the protective action of GHB against $H_2O_2$-evoked decreased viability or survival of native, control vector- and APPwt-transfected cells. More importantly, the combination of pharmacological investigations with flow cytometry analysis, reverse transcription, qPCR and Western blot experiments, which allowed the determination of GHB effects against APPwt-overexpression- or $H_2O_2$-induced apoptosis, also made it possible to get insights into the mechanisms activated by GHB to rescue native and genetically modified SH-SY5Y cells from oxidative stress-evoked death.

## EXPERIMENTAL PROCEDURES

### Chemicals and reagents

Dubelcco's modified eagle medium (DMEM), penicillin/streptomycin, hygromycin, $H_2O_2$, GHB, TRIzol reagent, Trypan Blue and MTT were purchased from Sigma (St. Quentin, France). Fetal calf serum was from Gibco (Basel, Switzerland). DNA Primers were from Eurogentec (Angers, France). iQ SYBR® Green Supermix and iScript cDNA Synthesis Kit were purchased from Biorad (Hercules, CA, USA). TUNEL Reagents were from Roche Diagnostics (Mannheim, Germany).

### Cell cultures

Human neuroblastoma SH-SY5Y cells were grown at 37 °C under an atmosphere of 5% $CO_2$ in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mm Glutamax and 1% (v/v) Penicillin/Streptomycin. SH-SY5Y cells were stably transfected with DNA constructs harboring human wild-type $APP_{695}$ (APPwt) or the expression vector pCEP4 (Invitrogen, Saint Aubin, France) alone (control vector) using lipofect AMINEplus (Invitrogen, Saint Aubin, France) (Scheuermann et al., 2001). Transfected APPwt cells were grown in DMEM standard medium supplemented with 300 μg/ml hygromycin.

### Trypan Blue exclusion assays

Cells were seeded into a 24-well plate ($10^5$ cells per well) and incubated for 48 h at 37 °C under an atmosphere of 5% $CO_2$ in DMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum. Native, control vector-pCEP4- and APPwt-transfected SH-SY5Y cells were treated with various concentrations of $H_2O_2$ ranging from 0 to 1 mM. After 24 h, cells were detached from the plate using a 0.05% trypsin-EDTA solution and immediately after DMEM supplemented with serum was added. Equal volumes of the cell suspension and 0.4% (v/v) Trypan Blue in phosphate-buffered saline (PBS) were mixed. Ten microliters of each mixture was transferred to a non-gridded disposable Countess® chamber Slide and the cells were scored using a Countess® automated cell counter (Invitrogen). Each probe was counted twice. Percent of cell survival was calculated by the Countess® software (Invitrogen) as the number of living cells divided by total cell number (including dead and living cells).