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Affinity of Bacterial Produced Poly (γ-Hydroxybutyrate) Nanoparticles to GHB Sites in Rat Brain

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Abstract

Nanotechnology is an emerging field that has been recognized to have the potential to make impacts on the prevention, detection and treatment of different diseases and disorders. Polymeric Nanoparticles (NPs) represent one of the most innovative non-invasive approaches for the drug delivery to the Central Nervous System (CNS). The NPs have the ability to cross the Blood Brain Barrier (BBB), thus allowing the drugs to exert their pharmacological activity in the central nervous district. In this study, we described that structurally similar bacterial produced P(γ-HB) polymer to that of naturally produced GHB showed similar effects in the expression of GHB mediated pathways. The hypothesis was tested *in vivo* using Sprague Dawley rats by treating the rats with P(γ-HB)-mPEG polymer in the form of NPs. Treatment of rats with P(γ-HB)-mPEG NPs leads to an inhibitory expression of dopamine D_1R, dopamine D_2R, $GABA_{B1}R$, and $GABA_{B2}R$ in the hippocampus relative to vehicle treated rats as demonstrated by Western blot analysis. This work also clearly demonstrates a dose dependent reduction of respective proteins as 50 mg/kg of P(γ-HB)-mPEG NPs showed more reduced expression of dopamine D_1R, dopamine D_2R, $GABA_{B1}R$, $GABA_{B2}R$ compared to that of 25 mg/kg P(γ-HB)-mPEG NPs. This new approach for targeting $GABA_B$ receptors using particulate system may provide a new insight for the design of drugs for the future therapies of various neurological disorders and other CNS diseases like cataplexy associated with narcolepsy.

Keywords: Gamma-Aminobutyric Acid (GABA); Gamma-Hydroxybutyric Acid (GHB); Narcolepsy; Nanoparticles; P(γ-HB)

Introduction

Gamma-Hydroxybutyric Acid (GHB) is a short chain fatty acid that occurs naturally in mammalian CNS [1,2]. GHB is formed in the brain from γ-Aminobutyric Acid (GABA)-derived succinic semialdehyde via succinic semialdehyde reductase [3]. GHB play a role in the brain as neurotransmitter or neuromodulator. GHB is a registered drug for the treatment of cataplexy and narcolepsy, and has also showed therapeutic efficacy for treating drug and alcohol dependence [4,5]. Any perturbation in the GABA pathway result in most common disorders such as epilepsy and other neuronal disorders which are mediated through GHB [6]. Therefore, exogenous administration of GHB was observed to produce more pronounced effects and play a significant role to cover the gaps of endogenously produced GHB [1,3]. Poly(γ-hydroxybutyrate) (P(γ-HB)) is more suitable candidate to be utilized in pharmaceutical industries due to its wide medical applications [7]. P(γ-HB) is a homopolymer of γ-hydroxybutyrate (γ-HB), and belongs to a diverse class of materials called polyhydroxyalkanoates (PHAs) produced by microorganisms inside the cells as energy storage materials [8]. P(γ-HB) has certain unique properties such as

biocompatibility and rapid *in vivo* degradation, which differentiate it from others PHA based polymers [9,10]. The products formed after degradation of P(γ-HB) are monomers and oligomers of GHB in which the monomers act as a natural human metabolite and play an important role in brain function [11]. The metabolite resulted after degradation of P(γ-HB) has short half-life (~35 min), therefore, eliminated easily form the body via Krebs cycle [12]. Several studies described that nanoparticles preparation from PHA-mPEG copolymer has shown no toxicity when evaluated *in vitro* models of animal's study [13,14]. Moreover, nanoparticles preparations from such polymer were also shown to be internalized in sufficient amount by human prostate cancer cell line [15].

P(γ-HB) has structural similarity to endogenously produced gamma-hydroxybutyric acid in the brain of animals. The difference is only in the length of monomer units which is repeated in the case of P(γ-HB), while a single monomer unit is present in the natural endogenous produced one. GHB is produced endogenously and also administered exogenously for the supplementation of intracellularly produced GHB by GABA in the brain. Exogenously administered GHB concentration was suggested to produce more pronounced neuronal functions than endogenously synthesized [3]. However, the major problem associated with exogenously administration of GHB is its use as abuse drug; producing mild euphoria, muscle relaxation, sedation, and eventually coma with increasing dosage [6,16]. In our study we suggest that P(γ-HB) a homologue of GHB, may induce similar effect in the induction of GABA mediated proteins as does GHB. However, the general structural similarities do not necessarily associate always with the common reaction mechanism. This study was hypothesized to find out any connection of bacterial produced P(γ-HB) with that of naturally produced GHB in the induction of GABA mediated pathways. P(γ-HB) an analogue of GHB was presumed to produced similar effects as that of exogenously administered GHB. Moreover, exogenously administration of P(γ-HB) is suggested to solve the problem associated with the treatment of narcoleptic patient with GHB such as drug abuse.

Materials and Methods

Preparation of P(γ-HB)-mPEG polymer

P(4HB) homopolymer was produced using the method of three-step cultivation of *Hydrogenophaga pseudoflavia* ATCC 33668 [17]. The nutrient broth-grown cultures were transferred to the Luria-Bertani medium and cultivated for 22 h. The cells isolated by centrifugation were incubated in the carbon-free mineral medium (the same mineral composition as in the PHA synthesis medium) containing 0.3 or 0.6 g /L ammonium sulfate for 10 h [18]. The cells recovered from the second cultivation step were transferred to the nitrogen-free PHA synthesis medium containing 2.24 g/L of γ-butyrolactone and cultivated for 36 h. The polyester was extracted with hot chloroform in a Pyrex Soxhlet apparatus. The mPEG [number average molecular weight (M_n) = 5000], bis(2-ethylhexanoate) tin catalyst, sodium deoxycholate and Dichloromethane (DCM) were purchased from Sigma-Aldrich Korea Ltd. All other chemicals used were of analytical grades. The synthesis of P(γ-HB)-mPEG diblock copolymer was carried out by transesterification method in the melt as previously described [15]. P(γ-HB)-mPEG blank nanoparticles were prepared from the diblock copolymer by a modified emulsification-solvent evaporation method [15].

Physiochemical Properties of P(γ-HB)-mPEG NPs

Morphological observation of nanoparticles was carried out using field emission scanning electron microscopy (FE-SEM Model XL30S, Philips, The Netherlands). For FE-SEM observation the nanoparticles were fixed in a 2.5% glutaraldehyde solution for 2h. The samples were washed twice with PBS buffer and then postfixed overnight in 1% osmium tetraoxide. The samples were again washed twice with PBS and dehydrated in a series of ethanol solutions (10%, 50%, 70%, 90%, 95%, 100%) and dried. For the morphological examination of samples for FE-SEM, the dried nanoparticles were dropped onto a carbon studs with carbon tape. The nanoparticles were coated with gold by a sputter coater (JFC-1100E, ion sputtering device, JEOL, Japan) for 300 s under vacuum at a current intensity of 10 mA and observed under the microscope. Particle sizes and size distributions were determined by the light scattering method (DLS-8000; Otsuka Electronics Co., Osaka, Japan). The mean particle size of the NPs was determined in triplicate, and the average values were calculated. The surface charge of the NPs was characterized in terms of zeta potential, which was determined using electrophoretic light scattering (ELS-Z; Otsuka Electronics Co., Osaka, Japan) at a scattering angle of 20°.

Animal Grouping and Drug Treatment

Adult male Sprague-Dawley rats weighing 250 g (n = 6) were housed individually in 45 x 24 x 20 cm plastic cages containing rodent bedding in a colony room maintained on a 12:12 light/dark cycle. Water was available continuously in the home cage. Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, Gyeongsang National University, South Korea. Two different doses of P(γ-HB)-mPEG nanoparticles (25 and 50 mg/kg) was chosen for chronic administration once daily for 14 days intraperitoneally. The P(γ-HB)-mPEG nanoparticles were suspended in sterile saline and sonicated for 10 min before injection in order to make a homogenous suspension. Control rats were injected with blank saline. After 14 days the rats were anesthetized with diethyl ether and subsequently killed. Brain sections were removed almost 14 h after the last injection. The brain portions were frozen on dry ice

and stored at -70°C until use for Western blot analysis.

Western Blot Analysis

Western blot analyses were performed according to the previously determined procedures [19]. The brain tissues (cortex and hippocampus) were gently homogenized in 0.2 M PBS containing protease inhibitor cocktail. The homogenate was centrifuged and the supernatant was used for the measurement of protein concentration. The protein concentration was measured by Bradford assay with the Bio-Rad protein assay solution. Proteins (30 µg) were separated on a 12.5% SDS-PAGE and transferred onto a Polyvinylidene Difluoride (PVDF) membrane (Millipore Corporation). Prestained protein marker, broad range: 7-200 kDa (Color plus pre-stained marker, EBM-1035, Elpis, Korea) was run in parallel for detection of the molecular weights of the proteins. Skimmed milk was used for membrane blocking as to reduce non-specific binding. The immunoblotting was performed using rabbit derived $GABA_BR1$, $GABA_BR2$, Dopamine D_1/D_2R, polyclonal antibodies (1:500; Santa Cruz Biotechnology, Cell signaling). Rabbit derived anti-β-actin antibody (1:500; Sigma, St. Louis, MO, USA) was taken as control to confirm uniform loading. The membranes were probed with a goat derived horseradish peroxidase-conjugated anti-rabbit IgG (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing with 1 x TBST, the blots were developed with super signal Enhanced Chemiluminescence (ECL) detection system (Amersham Biosciences, Piscataway, NJ, USA). The X-ray films were scanned and optical densities of the bands were measured using computer based Sigma Gel software (Jandel Scientific, San Rafeal, Chicago, USA).

Data Analysis and Statistics

The object bands of Western blot were scanned and analyzed by densitometry using a computer based on program on Sigma Gel System (SPSS Inc., Chicago, IL). Data were presented as mean ± standard error of mean (SEM). Data were analyzed by ANOVA followed by t-test. A level of $p < 0.01$ and $p < 0.05$ was considered to be significant. '#' indicates significant difference ($p < 0.01$), while '*' indicates significant difference ($p < 0.05$).

Results

Structural Resemblance of P(γ-HB) with GHB

P(γ-HB) has structural similarity to endogenously produced gamma-hydroxybutyric acid in the brain of animals. The difference is only in the length of monomer units which is repeated in the case of P(γ-HB), while a single monomer unit is present in the natural endogenous produced GHB (Figure 1).

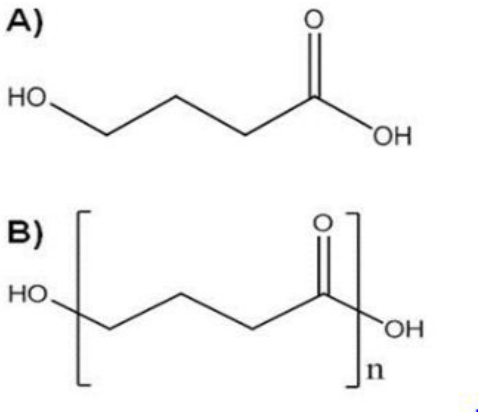

Figure 1: Structural resemblance of (A) gamma hydroxybutyrate and (B) poly-gamma hydroxybutyrate.

Characterization of P(γ-HB)-mPEG Nanoparticles

P(γ-HB)-mPEG nanoparticles were prepared by emulsification-solvent evaporation technique. Morphological examination under Scanning Electron Microscopy (SEM) showed the formation of smooth spherically shaped nanoparticles as described in (Figure 2).

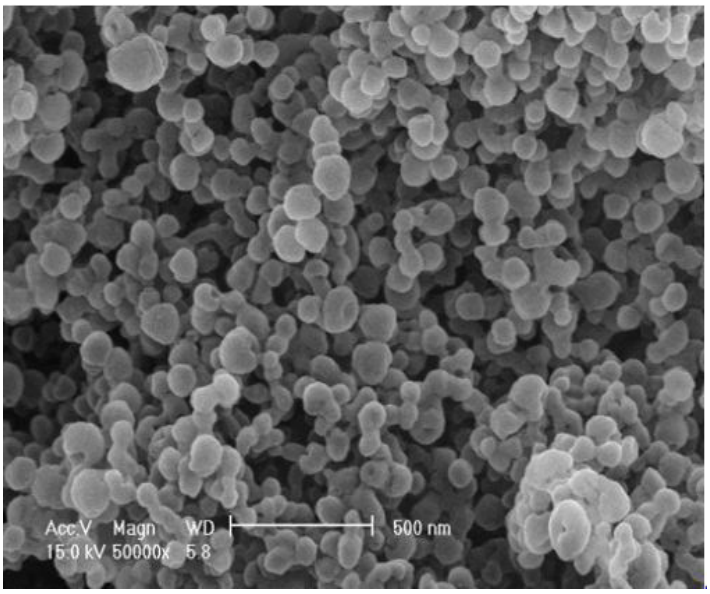

Figure 2: Morphological characterization of P(γ-HB)-mPEG NPs.

The SEM image shows the formation of spherical rounded shaped nanoparticles. The size of the nanoparticles observed through DLS was 110 nm and zeta potential as observed by ELS was -19 mV (data not shown).

Effect of P(γ-HB)-mPEG Nanoparticles on Expression of Dopamine D_1R and Dopamine D_2R in the hippocampus of Adult Rats

Previous studies have shown that acute treatment of GHB inhibited the dopamine release [20]. We determined the duration of effect of the nanoparticles in the expression of proteins in hippocampus regions of the rat brain. We chose chronic administration of the nanoparticles because the P(γ-HB) polymer is degraded slowly in nature, however a rapid degradation was suggested *in vivo* producing the monomer units of γ-HB a potential brain metabolite within given time frame [9,10]. As shown in (Figure 3), repeated exposure to P(γ-HB) induced a significant lower expression of dopamine D_1 and D_2 receptors in the hippocampus region of rat brain as compared to vehicle treated rats (control rats).

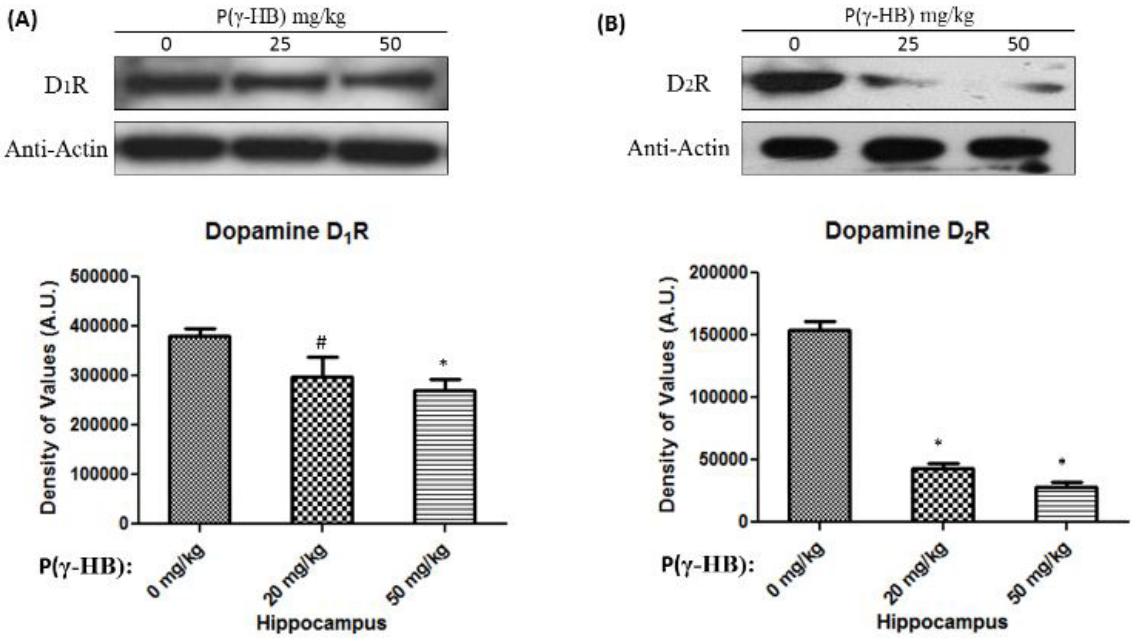

Figure 3: P(γ-HB)-induced decrease expression of dopamine receptors D_1R and D_2R in in hippocampal regions of rat brain. A: Representative Western blot analysis with dopamine D_1R antibody. Mean ± SEM (n=3) of the density of the corresponding proteins of dopamine D1R are presented. B: Representative Western blot analysis with dopamine D_2R antibody. Mean ± SEM (n = 3) of the density of the corresponding proteins of dopamine 2 are presented. Dopamine D1/D2 expression was significantly inhibited in hippocampus following chronic administration of P(γ-HB). *$p < 0.05$ and #$p < 0.01$ versus control group.

Effect of P(γ-HB)-mPEG Nanoparticles on Expression of $GABAB_1R$ and $GABAB_2R$ in the Hippocampus of Adult Rats

Next, we determine the effect of P($_\gamma$-HB)-mPEG nanoparticles on the protein expression level of $GABAB_1R$ and $GABAB_2R$ in the hippocampus of rat brain. We observed a significant decreased expression of $GABAB_2R$ with either dose i.e. 25 mg/kg or 50 mg/kg in the hippocampus region of rat's brain. However, $GABAB_1R$ showed significant decrease in the protein expression with chronically treated higher dose (50 mg/kg) of P(γ-HB) mPEG nanoparticles (Figure 4).

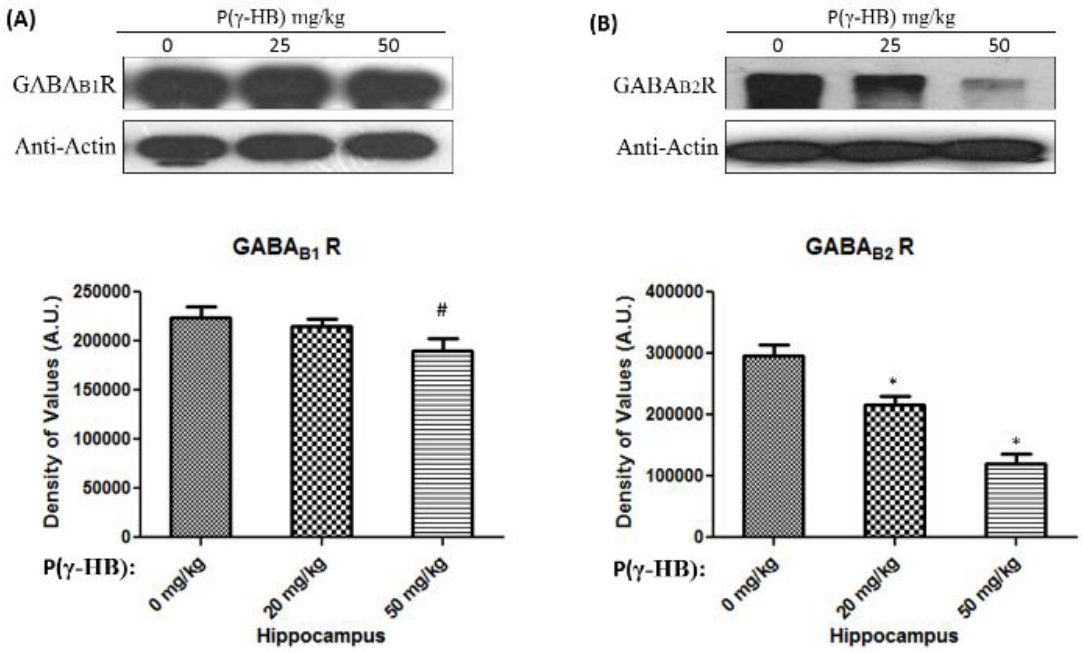

Figure 4: P(γ-HB)-induced decrease expression of GABA receptors $GABA_{B1}R$ and $GABA_{B2}R$ in the hippocampal regions of rat brain. A: Representative Western blot analysis with anti-$GABA_{B1}$. Mean ± SEM (n=3) of the density of the corresponding proteins. B: Representative Western blot analysis with anti-$GABA_{B2}$. Mean ± SEM (n=3) of the density of the corresponding proteins. $GABA_{B1}R$ and $GABA_{B2}R$ expression was significantly inhibited in hippocampus following chronic administration of P(γ-HB). * $p < 0.05$ and # $p < 0.01$ versus control group.

No toxicity with mPEG modified P(γ-HB) nanoparticles was observed in all the groups treated nanoparticles in our work (data not shown).

Discussion

GHB is a putative neurotransmitter or neuromodulator in mammalian brain [21, 22]. Exogenously administered it produces a number of physiological, pharmacological and biochemical effects in brain, including anxiolytic effect, sleep, anesthesia and absence seizures [22-24]. Moreover, GHB modifies the levels of different neurotransmitters, most notably dopamine, opioids, glutamate and acetylcholine [21,25,26]. Sufficiently large doses of the GHB metabolite have been approved by FDA for the treatment of cataplexy attacks in patients with narcolepsy [9]. Most of the known physiological effects of GHB have been demonstrated to be a result of either direct or indirect $GABA_B$ receptor activation [27,28]. However, the exact mechanism how the GHB exerts its effect in the CNS is still not clear.

In this study we demonstrated the modulating effect of P(γ-HB)-mPEG nanoparticles on dopamine and GABAB receptors. Treatment of rats with P(γ-HB) polymer in the form of nanoparticles leads to the suppression of dopamine D_1R and D_2R, and $GABA_{B1}R$ and $GABA_{B2}R$, in the hippocampus as demonstrated by Western blot analysis. The prepared nanoparticles have the ability to remain suspended in aqueous solution without forming aggregate. Such type nanoparticles have already been described to cross the blood brain barrier [29-31]. The mPEG used in the study is nontoxic and approved by FDA for human use [32]. Moreover, surface modification of the polymer with mPEG provide water dispersibility for the P(γ-HB) polymer thus prevent its precipitation [33]. Particulate system is mostly preferred as drug delivery vehicle and most of the hydrophobic drugs are currently tested for their improved therapeutic efficacy [34,35]. In this system the particle itself is supposed to show therapeutic effect without loading of any foreign object that will elicit a response mediated by endogenously produced or exogenously administered GHB. The exact mechanism of P(γ-HB) polymer expression of GHB mediated proteins is not clearly understood. However, most probably the P(γ-HB) polymer is degraded in to its monomer units and then cross the blood brain barrier to reach its target site.

Previously it was also suggested that P(γ-HB) degraded *in vivo* rapidly to produce the monomer units of γ-HB a potential brain metabolite within given time frame [9,10]. On the other hand, some research studies described that the polymer may enter the brain as whole in the form of particulate system and elicit the response [29-31]. However, in our study no such accumulation of nanoparticles in the brain parts was observed. So, it can be clearly concluded that the nanoparticles enter the brain in the form

degraded monomer units and elicit a response. Our hypothesis demonstrates that the receptors for GHB and P(γ-HB) are the same and they belong to the same family of macromolecular complexes and that they share structural similar ligands for modulation. It has already known that GHB has affinity for two distinct binding sites in the brain, the GHB receptor and, at higher concentrations, the $GABA_B$ receptor sites [36,37].

A recent study by Snead indicates that GHB receptor, like $GABA_B$ receptor, is coupled to a G protein, but it differs from $GABA_B$ receptor, having significantly different anatomical distribution and ontogeny [38,39]. The putative GHB receptor antagonist NCS-382, and different $GABA_B$ agonists and antagonists have been used to discriminate the relative role of GHB and $GABA_B$ receptors, and to understand the functional meaning of endogenous GHB. NCS-382 has been reported to antagonize some effects of GHB such as GHB-induced seizures and GHB self-administration in mice [23,24,40]. Moreover, novel cyclic γ-Hydroxybutyrate (GHB) analogs were synthesized which showed high affinity and stereoselectivity of binding to GHB sites in rat brain [16,41]. Structurally similar molecules to GHB receptor ligand were found to be associated with similar induction of response as that of GHB mediated such as the implication of sleep process was found to be similarly induced by cannabis receptors [42].

As earlier come in our discussion, there are structural analogy between GHB and P(γ-HB) therefore, for both of these brain metabolites it is necessary to decipher the possible role in the activation GH level and their interactions with their receptors. Moreover, it would be easier to develop new compositions and methods to deliver therapeutic amounts of GHB *in vivo* that may reduce the side effects associated with direct administration of GHB. In order to prove whether it could be used as chemotherapeutic agent for narcolepsy or other alcohol withdrawal, more **comprehensive** research and clinical trials have to be done.

Conclusion

In this paper, a new insight of research is proposed to correlate any connection of naturally occurring GHB with bacterial produced P(γ-HB). This new approach for targeting GABA receptors using particulate system of P(γ-HB)-mPEG nanoparticles may provide a major breakthrough for the design of drugs for the therapy of narcolepsy, absence seizures, drug addiction and withdrawal etc. Furthermore, the present study strengthens previous reports on the GHB activation of GABA associated proteins and will help focus future studies related to similar polymers. Our findings represent the first report that GHB markedly decrease expression of $GABA_{B2}$, dopamine D_1, D_2 in hippocampus via $GABA_B$ receptor-mediated pathway. These findings have revealed totally novel action of P(γ-HB) and thus provide a new insight into its effect on the CNS. The novel technology can be extended to additional agents, so as to target multiple signaling pathways thus providing a major breakthrough as a therapeutic modulator for various diseases.

Future perspectives

This new approach for targeting GABA receptors using particulate system may provide a new insight for the design of drugs for the future therapy. Furthermore, the present study may strengthen the previous reports on the GHB activation of GABA associated proteins and will help focus future studies related to similar polymers. The novel technology can be extended to additional agents, so as to target multiple signaling pathways providing a major breakthrough as a therapeutic modulator for various diseases. In order to prove whether it can be used as chemotherapeutic agent for narcolepsy or alcohol withdrawal, more **comprehensive** research and clinical trials have to be done.

Declaration of Interest

The authors declare no conflict of interest.

Ethical Conduct of Research

The authors state that they have obtained appropriate institutional review board approval. Animals were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee, Gyeongsang National University, South Korea.

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