Neuroscience 141 (2006) 269–275

γ-HYDROXYBUTYRATE INDUCES CYCLIC AMP-RESPONSIVE ELEMENT-BINDING PROTEIN PHOSPHORYLATION IN MOUSE HIPPOCAMPUS: AN INVOLVEMENT OF $GABA_B$ RECEPTORS AND cAMP-DEPENDENT PROTEIN KINASE ACTIVATION

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Abstract—γ-Hydroxybutyrate is a widely used recreational drug. Its abuse has been associated with cognitive impairments and development of tolerance and dependence. However, the neural mechanisms underlying these effects remain unclear. In the present study we investigated the possible cellular signaling mechanisms that might mediate γ-hydroxybutyrate's action. Acute administration of γ-hydroxybutyrate (500 mg/kg, i.p.) was found to cause a rapid and long-lasting increase in the phosphorylation level of the cAMP-responsive element-binding protein in mouse (C57/BL6) hippocampus. Pretreatment with the specific $GABA_B$ receptor antagonist [3-[1-(R)-[(3-cyclohexylmethyl)hydroxyphosphinyl]-2-(S)-hydroxy-propyl]amino] ethyl]-benzoic acid (20 mg/kg, i.p.) prevented the action of γ-hydroxybutyrate, confirming a $GABA_B$ receptor-mediated mechanism. In addition, acute γ-hydroxybutyrate administration induced a significant increase in cytosolic cAMP-dependent protein kinase activity in the hippocampus, and pretreatment with the cAMP-dependent protein kinase inhibitor H-89 could prevent the effect of γ-hydroxybutyrate on cAMP-responsive element-binding protein phosphorylation, indicating a direct involvement of cAMP-dependent protein kinase in γ-hydroxybutyrate-induced cAMP-responsive element-binding protein phosphorylation. On the other hand, the increased expression of phosphorylated cAMP-responsive element-binding protein was not observed in the hippocampus of mice subjected to repeated γ-hydroxybutyrate exposure, suggesting the development of a γ-hydroxybutyrate-induced desensitization of the signaling pathway leading to cAMP-responsive element-binding protein activation. Since cAMP-responsive element-binding protein activation has been implicated in a variety of neural plasticities, our findings may have revealed a new mechanism underlying γ-hydroxybutyrate-induced neuroadaptations. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: γ-hydroxybutyric acid, GABA receptors, mechanism, signal transduction, neuroadaptation.

γ-Hydroxybutyric acid (GHB) is a metabolite of GABA. It has been suggested to function as a neurotransmitter or neuromodulator (Maitre, 1997; Bernasconi et al., 1999; Snead and Gibson, 2005), although the precise physiological role of endogenous GHB is unknown. Peripherally administered GHB has been observed to produce significant behavioral, electrophysiological, and biochemical effects. Clinically, GHB is used as an anesthetic agent (Kleinschmidt et al., 1999), for the treatment of narcolepsy (Tunnicliff and Raess, 2002), and against alcohol and opiate dependency (Gallimberti et al., 1989; Addolorato et al., 1999).

However, GHB has recently emerged as a new recreational drug of abuse because of its euphoric and sedative effects, and its reputed anabolic actions (Nicholson and Balster, 2001; Wong et al., 2004). Besides the immediate behavioral problems resulting from GHB exposure, an acute overdose of GHB can cause coma, seizures, vomiting and even death (Galloway et al., 1997; Bernasconi et al., 1999; Snead and Gibson, 2005), whereas long-term use of GHB may lead to the development of tolerance and physical dependence in humans and has been associated with a withdrawal syndrome (Galloway et al., 1997; Nicholson and Balster, 2001; Miotto et al., 2001; McDonough et al., 2004; Snead and Gibson, 2005). The tolerance to and dependence on GHB or its metabolic precursor γ-butyrolactone have also been reported in several animal models, including rats, mice and baboons (Itzhak and Ali, 2002; Bania et al., 2003; Van Sassenbroeck et al., 2003; Weerts et al., 2005). Chronic GHB was also reported to develop cross-dependence with ethanol (Colombo et al., 1995). GHB produces conditioned place preference and is readily self-administered in animals (Martellotta et al., 1997; Fattore et al., 2000, 2001), indicating its rewarding properties. The development of tolerance and dependence suggests the occurrence of the adaptive change(s) in GHB-induced neuronal structures and functioning. In addition to the addictive properties, exogenously administered GHB may directly affect the cognitive processes. For example, GHB produces memory deficits in humans (Galloway et al., 1997; Wong et al., 2004). So far, the knowledge of cellular and molecular mechanisms related to GHB-induced neural changes remains very poor.

The nuclear transcription factor cyclic AMP-responsive element-binding protein (CREB) is widely accepted as one of the prototypical stimulus-inducible transcription factors. CREB activation has been shown to be altered in response to several of abused drugs, including stimulants and ethanol, suggesting that it may play a role in the neuroadap-

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Abbreviations: CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; CGP56999A, [3-[1-(R)-[(3-cyclohexylmethyl)hydroxyphosphinyl]-2-(S)-hydroxy-propyl] amino]ethyl]-benzoic acid; CREB, cyclic AMP-responsive element-binding protein; GHB, γ-hydroxybutyrate; MAP kinase, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase.

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doi:10.1016/j.neuroscience.2006.03.056

tive changes associated with the development of drug addiction (Blendy and Maldonado, 1998; Pandey et al., 2001; Lonze and Ginty, 2002; Noda and Nabeshima, 2004; Nestler, 2004; Carlezon et al., 2005). Based on these considerations, we investigated the effect of acute or chronic GHB on CREB (Ser133) phosphorylation in the hippocampus, a brain region with the highest level of endogenous GHB and a key brain structure implicated in a variety of neural plasticities including drug-induced response and memory (Nestler, 2004). We further examined whether cAMP-dependent protein kinase (PKA) was involved in this process.

EXPERIMENTAL PROCEDURES

GHB treatment

Six- to 10-week-old C57/Bl6 mice (Harlan, Indianapolis, IN, USA) were used. The doses of various drugs, including GHB (500 mg/kg, sodium salt, Sigma, St. Louis, MO, USA), (±)-baclofen (20 mg/kg, Sigma), CGP56999A ([3-[1-(R)-[(3-cyclohexylmethyl)hydroxyphosphinyl]-2-(S)-hydroxy-propyl] amino]ethyl]-benzoic acid, 20 mg/kg, kindly provided by Dr. Wolfgang Froestl, Novartis, Basel, Switzerland) were chosen based on our previous report (Ren and Mody, 2003). For acute GHB treatment, the mice were injected with GHB (i.p.) only once. For chronic GHB treatment, the mice were injected (i.p.) with GHB twice a day for 14 days. Some mice were subjected to 1, 3 or 5 days of withdrawal after the last injection. Control mice received saline. Following GHB injection or saline injection, the mice were killed by decapitation at indicated time points and the brains were rapidly dissected, frozen on dry ice, and kept at −80 °C until use for Western blot analysis.

For the i.c.v. injection of PKA inhibitor H-89 dihydrochloride (N-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide, 2HCl, Calbiochem, San Diego, CA, USA), mice were anesthetized and positioned on a stereotaxic frame with ear-bars plugged and jaws fixed to a biting plate. A small incision was made in the skin above the skull along the midline. The bregma point was identified and a hole was drilled with the following coordinates: 0.3 mm posterior and 1.0 mm lateral relative to bregma, and 3.1 mm down the skull surface. For each mouse, 2 μl of H-89 (10 mM in DMSO) or vehicle (DMSO) was injected at a rate of 1 μl/min. GHB or saline was given (i.p.) 20 min later. The animals were allowed to survive for another 30 min and killed by decapitation. The brain samples were dissected out and kept for further analysis.

Western blot analysis

Western blot analysis followed our previous report (Ren and Mody, 2003). Briefly, the hippocampi or hippocampal slices were homogenized at 4 °C in a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 0.5 mM dithiothreitol and protein phosphatase inhibitors). The homogenate was centrifuged at 12,000×g for 25 min and the supernatant was examined for protein concentration (by using a Dc Protein Assay kit, Bio-Rad, Hercules, CA, USA) and phosphorylation analysis.

For the detection of phosphorylated CREB, 20–40 μg of total protein was loaded onto a 10% polyacrylamide gel and subsequently transferred to a nitrocellulose membrane (Osmonics Inc., Minnetomka, MN, USA), and blocked with 5% milk in TBS-T. Membrane was incubated with anti-phospho-CREB (Ser 133; rabbit polyclonal, 1:1000 dilution; Cell Signaling Technology, Beverly, MA, USA). The protein was visualized by ECL (Amersham, Piscataway, NJ, USA). For the detection of total CREB, the membrane was stripped and re-labeled with the primary antibody against total CREB (rabbit polyclonal, 1:1000 dilution; Cell Signaling Technology). In some experiments, the membrane was stripped again and labeled with the mouse monoclonal primary anti-β-actin antibody (Sigma) to determine whether the expression level of total CREB was affected by GHB treatment. All of our measurements about CREB phosphorylation are expressed as the ratio (*p*/*t*) of phospho-CREB (*p*) to total CREB (*t*).

For the analysis of phosphorylated PKA substrates, 5–10 μg of total protein was loaded, and an anti-phospho-PKA substrate (RRXS*/T*) (100G7) rabbit monoclonal antibody (1:5000 dilution; Cell Signaling Technology) was used. The relative level of the phosphorylated PKA substrate(s) was compared with β-actin.

PKA activity assay

To prepare the subcellular fractions, the hippocampal tissue was homogenized in homogenizing buffer (50 mM Tris–HCl, pH 7.5, 5 mM EDTA and10 mM EGTA) containing 0.5 mM dithiothreitol and protein phosphatase inhibitors. The homogenate was centrifuged at 100,000×g for 30 min at 4 °C. The supernatant was collected as cytosolic fraction, and the pellet was resuspended in above homogenizing buffer containing 1% (w/v) Triton X-100. The homogenate was kept at 4 °C for 60 min with occasional stirring and finally centrifuged at 100,000×g for 20 min at 4 °C. The resulting supernatant was used as the membrane fraction.

PepTag non-radioactive detection of PKA was performed according to the kit instructions (Promega, Madison, WI, USA). In this kit, phosphorylation of the PKA-specific substrate (peptide) was used to measure kinase activity. In our experiments, both cytosolic and membrane proteins (20 μg of protein were applied respectively) were used for PKA activity assay. After the reaction was terminated, phosphorylated and unphosphorylated PepTag peptides were separated on a 0.8% agarose gel. The gel was photographed with a transilluminator, and bands quantified by densitometry. The fluorescence intensity of peptide substrate specifically phosphorylated by PKA was measured as an indication of the PKA activity.

Data analysis

All data are presented as mean±S.E.M. The statistical significance was assessed by a one-way ANOVA followed by a post hoc Tukey's HSD test (Figs. 1, 2, 4 and 5) or by using the student's *t*-test (Fig. 3).

RESULTS

Acute GHB induces CREB phosphorylation in mouse hippocampus

We first examined the time course of CREB phosphorylation after the acute injection of GHB (500 mg/kg, i.p.). As shown in Fig. 1, a significant increase (more than two-fold) in the phosphorylation level of CREB was observed in the hippocampus of mice after GHB treatment. The effect of GHB on CREB phosphorylation was rapid; it could be detected as early as 15 min following GHB injection. The effect of GHB on CREB phosphorylation in the hippocampus lasted for up to 2 h following GHB injection.

Following the acute GHB administration, the total level of CREB protein expression in the hippocampus, as detected by an antibody against total CREB, was unaffected (as compared with β-actin level, data not shown).

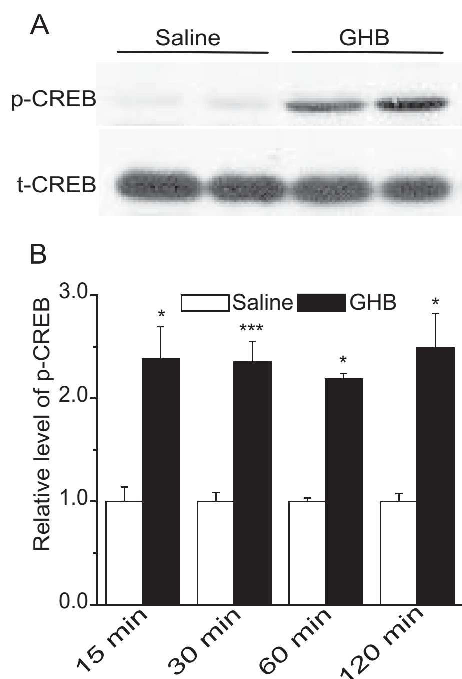

Fig. 1. Acute GHB administration significantly increases CREB phosphorylation in mouse hippocampus. (A) Representative Western blot detected with anti-phospho-CREB or anti-total CREB antibody in the tissues obtained 30 min following the injection of GHB (500 mg/kg, i.p.). (B) Exogenously administered GHB induces a rapid and long-lasting CREB phosphorylation in the hippocampus. The tissue was collected at the indicated time points following the injection of GHB (500 mg/kg, i.p.) or saline. *, *** $P<0.05$ or 0.001 vs. each one's control respectively ($n=3$–10 for each group).

GHB increases CREB phosphorylation via $GABA_B$ receptors

We next wanted to find out the role of $GABA_B$ receptors in the GHB-induced CREB phosphorylation. As shown in Fig. 2, a pretreatment with the specific $GABA_B$ receptor antagonist CGP56999A (20 mg/kg) administered 10 min before GHB prevented the stimulatory effect of GHB on CREB phosphorylation in the hippocampus, demonstrating that $GABA_B$ receptors are directly involved in increasing the CREB phosphorylation by GHB. In addition, unlike in the case of mitogen-activated protein kinase (MAP kinase) phosphorylation (Ren and Mody, 2003), CGP56999A alone, at the dose used (20 mg/kg, i.p.), failed to affect CREB phosphorylation in the mouse hippocampus.

We also used the selective $GABA_B$ receptor agonist baclofen as a positive control for $GABA_B$ receptor activation. Similar to GHB, acute application of baclofen (20 mg/kg, i.p.) significantly increased CREB phosphorylation in the hippocampus, further supporting the role of $GABA_B$ receptor activation in CREB phosphorylation.

PKA mediates GHB-induced CREB phosphorylation

cAMP signaling is proposed to be one of the major pathways leading to CREB activation (Lonze and Ginty, 2002). To test this possibility, we first determined whether PKA activity was altered in the hippocampus following acute GHB administration. The results showed that treatment of animals with acute GHB significantly increased cytosolic PKA activity, as compared with saline (Fig. 3A and C). However, the membrane-associated PKA activity was not affected by acute GHB administration (data not shown). In addition, we also used a phospho-PKA substrate antibody (Catalog No. 9624, Cell Signaling Technology) which detects PKA substrate peptides and proteins containing a phospho-Ser/Thr residue with arginine at the −3 and −2 positions. In particular, the phosphorylation level of a 75 kDa protein was found to be significantly increased following acute GHB administration (Fig. 3B and D).

To show a direct involvement of PKA activation in GHB-induced CREB phosphorylation in the hippocampus, we used H-89, a specific PKA inhibitor. In this experiment, the mouse was injected with H-89 (10 mM, 2 μl, i.c.v.) 20 min prior to GHB administration. Such a pretreatment was found to completely prevent the stimulatory effect of acute GHB on CREB phosphorylation (Fig. 4A and 4C), indicating that the increased PKA activity is required for GHB-induced CREB phosphorylation. In addition, it was found that pretreatment with H-89 could also prevent the phosphorylation of the 75 kDa protein detected with the phospho-PKA substrate antibody (Fig. 4B and 4D). The phosphorylation level of the 75 kDa protein in the hippocampus of GHB-treated animals was 164±14% of control, whereas the phosphorylation level in

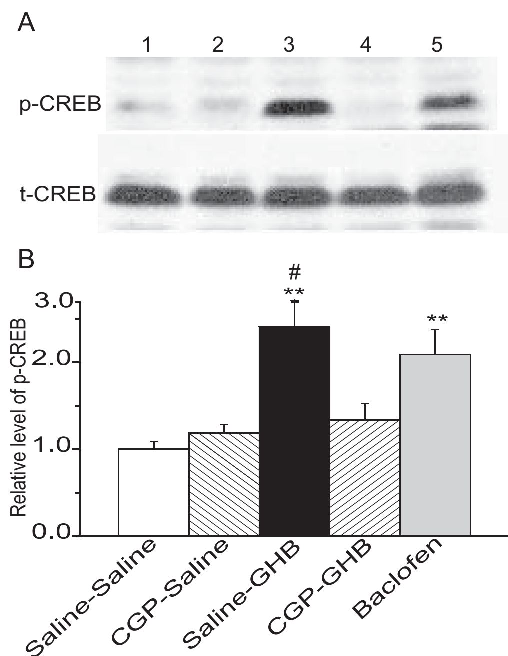

Fig. 2. The specific $GABA_B$ receptor antagonist CGP56999A reverses and the $GABA_B$ receptor agonist baclofen mimics the effect of GHB on CREB phosphorylation. (A) Representative Western blot detected with anti-phospho-CREB or anti-total CREB antibody. (B) Normalized phospho-CREB levels. CGP56999A (20 mg/kg, i.p.) was given 10 min prior to GHB or saline injection. The tissue was collected 30 min following the injection of GHB (500 mg/kg, i.p.), baclofen (20 mg/kg, i.p.) or saline. Lanes 1, 2, 3, 4 and 5 represent saline–saline, CGP56999A–saline, saline–GHB, CGP56999A–GHB and baclofen group respectively (CGP=CGP56999A, $n=5$ for each group). ** $P<0.01$ vs. saline–saline group; # $P<0.05$ vs. CGP56999A–GHB (CGP–GHB) group.

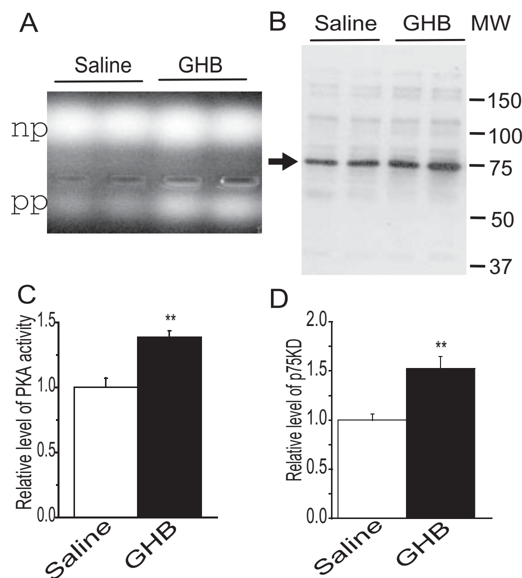

Fig. 3. Acute administration of GHB upregulates hippocampal cytosolic PKA activity. (A) Representative picture showing the phosphorylated peptide bands by PKA in saline- or GHB-treated hippocampal samples. (C) Relative level of PKA activity as quantitated by scanning densitometry of the phosphorylated peptide bands. (B) Representative Western blot detected with an anti-phospho-PKA substrate antibody. The arrow indicates a phosphorylated PKA substrate with a molecular weight of about 75 kDa. (D) Relative phosphorylation level of the 75 kDa PKA substrate (p75KD) after acute GHB treatment. The PKA activity was assayed by using a PepTag non-radioactive PKA assay kit. The tissue was collected 30 min following the injection of GHB (500 mg/kg, i.p.) or saline. ** $P<0.01$ vs. saline group ($n=5$ for each group, MW, molecular weight in kDa; np, non-phosphorylated peptide; pp, phosphorylated peptide).

hippocampus of H-89 plus GHB-treated animals was 109±14% of control ($n=4-6$).

In addition, we also examined the activations of MAP kinases (extracellular signal-regulated kinases 1 and 2, ERK1/2) and Ca^{2+}/calmodulin-dependent protein kinase (CaM kinase) IV, two other major protein kinases which have been proposed to be involved in the phosphorylation of CREB *in vivo* (Lonze and Ginty, 2002; Carlezon et al., 2005). It was found that the MAP kinase phosphorylation was significantly inhibited by acute GHB, confirming our previous findings (Ren and Mody, 2003), whereas the phosphorylation of CaM kinase IV was not affected by acute GHB administration (data not shown). These results suggest that MAP kinase or CaM kinase IV might play a minor role in GHB-induced CREB phosphorylation.

Taken together, these data show that acute GHB administration significantly increases the PKA activity, which is directly involved in the increased CREB phosphorylation in mouse hippocampus.

Effects of chronic GHB and its withdrawal on CREB phosphorylation

We assessed the effect of repeated GHB treatment and its withdrawal on CREB phosphorylation in mouse hippocampus. The chronic GHB model was established by injecting (i.p.) the mice with GHB (500 mg/kg) twice a day for 14

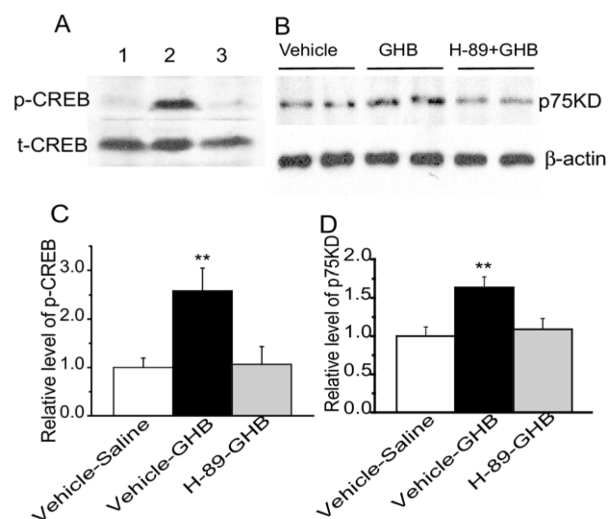

Fig. 4. GHB-induced CREB phosphorylation is prevented by pretreatment with the PKA inhibitor H-89. (A) Representative Western blot detected with anti-phospho-CREB or anti-total CREB antibody. (C) Normalized phospho-CREB levels. B and D show that H-89 can prevent the effect of GHB on phosphorylation of the 75 kDa PKA substrate (p75KD). H-89 (10 mM, 2 μl) or vehicle (DMSO, 2 μl) was given (i.c.v.) 20 min prior to GHB or saline injection. The tissue was collected 30 min after GHB (500 mg/kg, i.p.) or saline (i.p.) injection. ** $P<0.01$ vs. vehicle–saline group ($n=7$).

days. On the fifteenth day, the mice were given another injection. The brain samples were collected 30 min (chronic group), or 24 h (1 day withdrawal group), 72 h (3 days withdrawal group) or 120 h (5 days withdrawal group) respectively following the last injection. Some mice received one

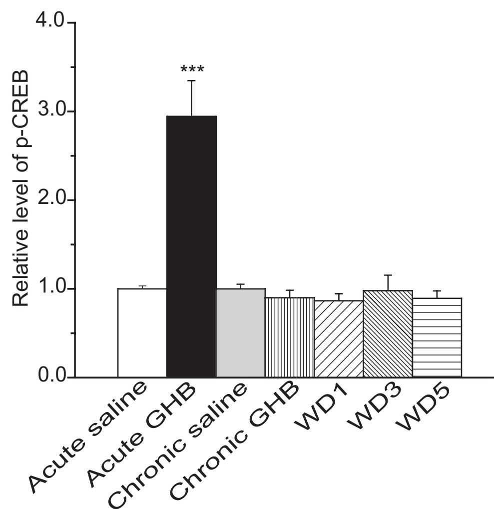

Fig. 5. The effect of chronic GHB administration and its withdrawal on CREB phosphorylation in the hippocampus. The mice were injected GHB (500 mg/kg, i.p.) or saline twice a day for 14 days. The brain samples were collected 30 min (chronic group), 1 day (WD1), 3 days (WD3) or 5 days (WD5) following the last injection respectively. The mice in acute GHB group received only one injection (GHB, 500 mg/kg, or saline), and the hippocampal tissues were collected 30 min following the injection. Normalized phospho-CREB levels were shown in this figure. *** $P<0.001$ vs. acute saline or any other experimental group (WD, withdrawal; $n=4-8$ for each group).

GHB (500 mg/kg) injection (acute GHB group). As can be seen in Fig. 5, although acute GHB could remarkably increase CREB phosphorylation, such a stimulatory effect of GHB on CREB phosphorylation was not detected in the hippocampus of mice that received repeated GHB administration for 14 days. Moreover, 1, 3, or 5 days of withdrawal from GHB did not produce any significant influence on the phosphorylation level of CREB either. These observations suggest that chronic GHB treatment may have caused a far-reaching effect on the intracellular signal pathway leading to CREB phosphorylation. In addition, we also measured the CREB phosphorylation resulting from chronic GHB and baclofen administration in another mouse model. In this model, mice were given GHB (500 mg/kg/day) or baclofen (20 mg/kg/day) in their liquid diet (Dyets Inc., Bethlehem, PA, USA) for 14 days. Similarly, no significant CREB phosphorylation was detected in the hippocampus of mice subjected to such chronic GHB or baclofen administration (105±13% and 112±8% of control, $P=0.74$ and 0.31 for GHB and baclofen respectively, $n=5$, *t*-test).

Finally, by using β-actin as an internal standard, we also compared the total CREB protein levels in the hippocampi of mice after receiving chronic GHB treatment or subjected to different days of GHB withdrawal. No difference in the total CREB levels was detected in these groups of mice (data not shown).

DISCUSSION

In present study, we demonstrate that acute GHB treatment (500 mg/kg) significantly increases the phosphorylation level of CREB in mouse hippocampus. This increase is mediated by the action of GHB on $GABA_B$ receptors. We further show that the activation of the cAMP signal pathway is involved in the stimulatory effect of GHB. In addition, we also find that chronic GHB administration can induce a long-term modulatory effect on the intracellular signaling pathway leading to CREB phosphorylation. These findings may provide new insights into the mechanisms of GHB-induced neuroadaptive changes such as addiction and memory impairments.

The finding that GHB induces CREB phosphorylation in the hippocampus is consistent with GHB's drug abuse mechanisms. GHB has been reported to cause tolerance and dependence in both humans and animal models (Galloway et al., 1997; Nicholson and Balster, 2001; Miotto et al., 2001; Itzhak and Ali, 2002; Bania et al., 2003; Van Sassenbroeck et al., 2003; Weerts et al., 2005; Snead and Gibson, 2005). However, no work is reported on the underlying mechanisms. As previously described, cAMP–CREB signaling pathway has been implicated in synaptic plasticity, including the response to several drugs of abuse (Blendy and Maldonado, 1998; Lonze and Ginty, 2002; Noda and Nabeshima, 2004; Ren et al., 2004; Nestler, 2004; Carlezon et al., 2005). CREB has been proposed to mediate a form of drug tolerance and dependence (Nestler, 2004). In the present study, we observed a significant increase in the phosphorylation level of CREB following acute GHB exposure in hippocampus, a brain structure that has recently been shown to play an important role in drug addiction (Pu et al., 2002; Nestler, 2004). In addition, our other preliminary work (unpublished observations) shows that acute GHB significantly increases CREB phosphorylation in mouse frontal cortex and striatum, two other brain structures critical for drug addiction (Nestler, 2004). These data support the hypothesis that CREB represents an important intracellular neuronal target that might be associated with mediating GHB-induced neuroadaptive changes in the CNS.

Our observation on CREB phosphorylation in the hippocampi of mice subjected to long-term GHB exposure supports the role of CREB phosphorylation in GHB-induced neuroadaptation. The mice subjected to repeated GHB administration showing no change in CREB phosphorylation levels in the hippocampus may implicate the occurrence of adaptation(s) in the intracellular signaling pathway(s) resulting from chronic GHB administration. In addition, withdrawal from chronic GHB does not alter CREB phosphorylation, unlike other drugs of abuse such as ethanol (e.g. Pandey et al., 2001). There might be several explanations for this. Firstly, it is likely that repeated GHB administration has resulted in a long neuroadaptation in the hippocampus. Secondly, it is also possible that we failed to pick up the right time point, as difference in the peaks of the behavioral withdrawal signs has been reported in different models (McDonough et al., 2004; Bania et al., 2003; Weerts et al., 2005). It cannot be excluded, however, that more prolonged treatment and/or higher doses of GHB may be needed to induce withdrawal syndrome in mouse. The molecular mechanisms underlying the effects of long-term GHB administration and withdrawal need further investigations.

GHB has been known for its amnesic effect in humans (Galloway et al., 1997; Wong et al., 2004) in addition to its addictive effects. Similarly, the systemic administration of baclofen has also been observed to induce memory deficits in rodents (e.g. Nakagawa et al., 1995). In the present study, we found that both GHB and baclofen could increase CREB phosphorylation in mouse hippocampus. Given the crucial role of the cAMP–CREB cascade in hippocampal-dependent memory processes (Lonze and Ginty, 2002), we propose that the fast induction of CREB phosphorylation by these $GABA_B$ receptor agonists might be implicated in GHB- or baclofen-induced memory impairments (Nava et al., 2001; Ferraro et al., 2001; Sircar and Basak, 2004).

Our work evidenced the involvement of $GABA_B$ receptor and PKA activation in GHB-induced CREB phosphorylation in the hippocampus. Although the receptor mechanism of GHB action remains controversial, most, if not all, pharmacological effects elicited by exogenously administered GHB, including hypothermia, the sedative/hypnotic effects and the properties of tolerance and addiction are mediated via $GABA_B$ receptors (e.g. Carai et al., 2001; Fattore et al., 2001; Kaupmann et al., 2003; Quéva et al., 2003; Carter et al., 2003; Goodwin et al., 2005). We have previously shown the role of $GABA_B$ receptors in mediating some effects of GHB (Jensen and Mody, 2001; Ren and

Mody, 2003). The present study, by using the specific $GABA_B$ receptor antagonist CGP 56999A and selective $GABA_B$ receptor agonist baclofen, demonstrated the involvement of $GABA_B$ receptors in GHB's stimulatory effect on hippocampal CREB phosphorylation. Furthermore, our work also shows a direct involvement of PKA in the $GABA_B$-mediated CREB phosphorylation. In addition to GHB, we also observed an increase in hippocampal PKA activity by baclofen (146±4% of control, $n=3$, $P=0.041$ by *t*-test. The tissues were obtained 30 min after the i.p. injection of baclofen at the dose of 20 mg/kg). Therefore, agonist activation of $GABA_B$ receptors in the hippocampus consistently up-regulates the cAMP/PKA pathway, at least under our experimental conditions. This finding is interesting, given that $GABA_B$ receptors, in most cases, have been observed to negatively couple to cAMP. Thus an activation of $GABA_B$ receptors will reduce PKA activity. It has been recorded, however, that $GABA_B$ receptor activation potentiated β-adrenergic agonist-induced cAMP accumulation in rat brain slices (Karbon and Enna, 1985) and a pre- or co-treatment with baclofen potentiated isoproterenol-induced increase of cAMP in rat striatum (Hashimoto and Kuriyama, 1997). Furthermore, in membranes of rat olfactory bulb, $GABA_B$ activation has been observed to stimulate adenylyl cyclase (type II/IV) activity (Olianas and Onali, 1999), which may be mediated through $\beta\gamma$ subunits of Gi/Go proteins. Such a stimulatory action of $GABA_B$ receptors on cAMP levels has been suggested to be a consequence of G protein crosstalk between $\beta\gamma$ subunits and Gs (Bettler et al., 2004). On the other hand, we would propose that the effect of GHB on the PKA activity (resulting from increased cAMP level) is likely to be a result of adenylyl cyclases I and/or VIII stimulation by Ca^{2+}/calmodulin. These adenylyl cyclases, expressed in hippocampus and directly stimulated by Ca^{2+} and CaM *in vivo*, have been implicated in several forms of synaptic plasticity in the hippocampus (Wang and Storm, 2003). In agreement with this hypothesis, Ito et al. (1995) demonstrated, in primary cultures of mouse cerebellar granule cells, that $GABA_B$ receptor stimulation by GHB significantly increased the intracellular Ca^{2+} concentration and meanwhile increased CRE-binding activity. Additional studies will be required on the precise mechanism of PKA stimulation mediated through GHB activation of $GABA_B$ receptors in the hippocampus.

CONCLUSION

In summary, this is the first study demonstrating that acute GHB stimulates phosphorylation of CREB via a $GABA_B$ receptor-mediated mechanism, which involves the activation of PKA. The phosphorylation of CREB may induce the transcription of numerous genes (such as c-fos) that contain a CRE in their promoter regions (Lonze and Ginty, 2002). In fact, a previous study by Zhang et al. (1991) has demonstrated the GHB-induced transcription of c-fos in the thalamus of a rat generalized absence epilepsy. In addition, we have, by using microarray analysis, identified a number of GHB-responsive transcripts including the immediate early gene c-fos (X. Ren and I. Mody, unpublished observations). These findings have added to our understanding of the molecular basis of GHB-induced neuroadaptations.

Acknowledgments—This work was supported by the National Institutes of Health grant DA14947 and the Coelho Endowment (to I.M.). The authors are grateful to Dr. Wolfgang Froestl for providing CGP56999A and John Chun Feng for expert help with the i.c.v. injection.

REFERENCES

Addolorato G, Balducci G, Capristo E, Attilia ML, Taggi F, Gasbarrini G, Ceccanti M (1999) γ-Hydroxybutyric acid (GHB) in the treatment of alcohol withdrawal syndrome: a randomized comparative study versus benzodiazepine. Alcohol Clin Exp Res 23:1596–1604.

Bania TC, Ashar T, Press G, Carey PM (2003) γ-Hydroxybutyric acid tolerance and withdrawal in a rat model. Acad Emerg Med 10: 697–704.

Bernasconi R, Mathivet P, Bischoff S, Marescaux C (1999) γ-Hydroxybutyric acid: an endogenous neuromodulator with abuse potential? Trends Pharmacol Sci 20:135–141.

Bettler B, Kaupmann K, Mosbacher J, Gassmann M (2004) Molecular structure and physiological functions of GABAB receptors. Physiol Rev 84:835–867.

Blendy JA, Maldonado R (1998) Genetic analysis of drug addiction: the role of cAMP response element binding protein. J Mol Med 76:104–110.

Carai MA, Colombo G, Brunetti G, Melis S, Serra S, Vacca G, Mastinu S, Pistuddi AM, Solinas C, Cignarella G, Minardi G, Gessa GL (2001) Role of $GABA_B$ receptors in the sedative/hypnotic effect of γ-hydroxybutyric acid. Eur J Pharmacol 428:315–321.

Carlezon WA Jr, Duman RS, Nestler EJ (2005) The many faces of CREB. Trends Neurosci 28:436–445.

Carter LP, Flores LR, Wu H, Chen W, Unzeitig AW, Coop A, France CP (2003) The role of $GABA_B$ receptors in the discriminative stimulus effects of γ-hydroxybutyrate in rats: time course and antagonism studies. J Pharmacol Exp Ther 305:668–674.

Colombo G, Agabio R, Lobina C, Reali R, Fadda F, Gessa GL (1995) Cross-tolerance to ethanol and γ-hydroxybutyric acid. Eur J Pharmacol 273:235–238.

Fattore L, Cossu G, Martellotta MC, Deiana S, Fratta W (2001) Baclofen antagonises intravenous self-administration of γ-hydroxybutyric acid in mice. Neuroreport 12:2243–2246.

Fattore L, Martellotta MC, Cossu G, Fratta W (2000) γ-Hydroxybutyric acid: an evaluation of its rewarding properties in rats and mice. Alcohol 20:247–256.

Ferraro L, Tanganelli S, O'Connor WT, Francesconi W, Loche A, Gessa GL, Antonelli T (2001) γ-Hydroxybutyrate modulation of glutamate levels in the hippocampus: an *in vivo* and *in vitro* study. J Neurochem 78:929–939.

Gallimberti L, Canton G, Gentile N, Ferri M, Cibin M, Ferrara SD, Fadda F, Gessa GL (1989) γ-Hydroxybutyric acid for treatment of alcohol withdrawal syndrome. Lancet 2:787–789.

Galloway GP, Frederick SL, Staggers FE Jr, Gonzales M, Stalcup SA, Smith DE (1997) γ-Hydroxybutyrate: an emerging drug of abuse that causes physical dependence. Addiction 92:89–96.

Goodwin AK, Froestl W, Weerts EM (2005) Involvement of γ-hydroxybutyrate (GHB) and $GABA_B$ receptors in the acute behavioral effects of GHB in baboons. Psychopharmacology 180:342–351.

Hashimoto T, Kuriyama K (1997) *In vivo* evidence that $GABA_B$ receptors are negatively coupled to adenylate cyclase in rat striatum. J Neurochem 69:365–370.

Ito Y, Ishige K, Zaitsu E, Anzai K, Fukuda H (1995) γ-Hydroxybutyric acid increases intracellular Ca2+ concentration and nuclear cyclic AMP-responsive element- and activator protein 1 DNA-binding activities through $GABA_B$ receptor in cultured cerebellar granule cells. J Neurochem 65:75–83.

Itzhak Y, Ali SF (2002) Repeated administration of γ-hydroxybutyric acid (GHB) to mice: assessment of the sedative and rewarding effects of GHB. Ann N Y Acad Sci 965:451–460.

Jensen K, Mody I (2001) GHB depresses fast excitatory and inhibitory synaptic transmission via $GABA_B$ receptors in mouse neocortical neurons. Cereb Cortex 11:424–429.

Karbon EW, Enna SJ (1985) Characterization of the relationship between gamma-aminobutyric acid B agonists and transmitter-coupled cyclic nucleotide-generating systems in rat brain. Mol Pharmacol 27:53–59.

Kaupmann K, Cryan JF, Wellendorph P, Mombereau C, Sansig G, Klebs K, Schmutz M, Froestl W, van der Putten H, Mosbacher J, Brauner-Osborne H, Waldmeier P, Bettler B (2003) Specific γ-hydroxybutyrate-binding sites but loss of pharmacological effects of gamma-hydroxybutyrate in $GABA_{B(1)}$-deficient mice. Eur J Neurosci 18:2722–2730.

Kleinschmidt S, Schellhase C, Mertzlufft F (1999) Continuous sedation during spinal anaesthesia: γ-hydroxybutyrate vs. propofol. Eur J Anaesthesiol 16:23–30.

Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605–623.

Maitre M (1997) The γ-hydroxybutyrate signaling system in the brain: organization and functional implications. Prog Neurobiol 51: 337–361.

Martellotta MC, Fattore L, Cossu G, Fratta W (1997) Rewarding properties of γ-hydroxybutyric acid: an evaluation through place preference paradigm. Psychopharmacology 132:1–5.

McDonough M, Kennedy N, Glasper A, Bearn J (2004) Clinical features and management of γ-hydroxybutyrate (GHB) withdrawal: a review. Drug Alcohol Depend 75:3–9.

Miotto K, Darakjian J, Basch J, Murray S, Zogg J, Rawson R (2001) γ-Hydroxybutyric acid: patterns of use, effects and withdrawal. Am J Addict 10:232–241.

Nakagawa Y, Ishibashi Y, Yoshii T, Tagashira E (1995) Involvement of cholinergic systems in the deficit of place learning in Morris water maze task induced by baclofen in rats. Brain Res 683:209–214.

Nava F, Carta G, Bortolato M, Gessa GL (2001) γ-Hydroxybutyric acid and baclofen decrease extracellular acetylcholine levels in the hippocampus via $GABA_B$ receptors. Eur J Pharmacol 430: 261–263.

Nestler EJ (2004) Molecular mechanisms of drug addiction. Neuropharmacology 47 (Suppl 1):24–32.

Nicholson KL, Balster RL (2001) GHB: a new and novel drug of abuse. Drug Alcohol Depend 63:1–22.

Noda Y, Nabeshima T (2004) Involvement of signal transduction cascade via dopamine-D1 receptors in phencyclidine dependence. Ann N Y Acad Sci 1025:62–68.

Olianas MC, Onali P (1999) $GABA_B$ receptor-mediated stimulation of adenylyl cyclase activity in membranes of rat olfactory bulb. Br J Pharmacol 126:657–664.

Pandey SC, Roy A, Mittal N (2001) Effects of chronic ethanol intake and its withdrawal on the expression and phosphorylation of the CREB gene transcription factor in rat cortex. J Pharmacol Exp Ther 296:857–868.

Pu L, Bao GB, Xu NJ, Ma L, Pei G (2002) Hippocampal long-term potentiation is reduced by chronic opiate treatment and can be restored by re-exposure to opiates. J Neurosci 22:1914–1921.

Quéva C, Bremner-Danielsen M, Edlund A, Ekstrand AJ, Elg S, Erickson S, Johansson T, Lehmann A, Mattsson JP (2003) Effects of GABA agonists on body temperature regulation in $GABA_{B(1)}^{-/-}$ mice. Br J Pharmacol 140:315–322.

Ren X, Mody I (2003) γ-Hydroxybutyrate reduces mitogen-activated protein kinase phosphorylation via $GABA_B$ receptor activation in mouse frontal cortex and hippocampus. J Biol Chem 278: 42006–42011.

Ren X, Noda Y, Mamiya T, Nagai T, Nabeshima T (2004) A neuroactive steroid, dehydroepiandrosterone sulfate, prevents the development of morphine dependence and tolerance via c-fos expression linked to the extracellular signal-regulated protein kinase. Behav Brain Res 152:243–250.

Sircar R, Basak A (2004) Adolescent γ-hydroxybutyric acid exposure decreases cortical N-methyl-D-aspartate receptor and impairs spatial learning. Pharmacol Biochem Behav 79:701–708.

Snead OC 3rd, Gibson KM (2005) γ-Hydroxybutyric acid. N Engl J Med 352:2721–2732.

Tunnicliff G, Raess BU (2002) γ-Hydroxybutyrate (orphan medical). Curr Opin Investig Drugs 3:278–283.

Van Sassenbroeck DK, De Paepe P, Belpaire FM, Boon PA, Buylaert WA (2003) Tolerance to the hypnotic and electroencephalographic effect of γ-hydroxybutyrate in the rat: pharmacokinetic and pharmacodynamic aspects. J Pharm Pharmacol 55:609–615.

Wang H, Storm DR (2003) Calmodulin-regulated adenylyl cyclases: cross-talk and plasticity in the central nervous system. Mol Pharmacol 63:463–468.

Weerts EM, Goodwin AK, Griffiths RR, Brown PR, Froestl W, Jakobs C, Gibson KM (2005) Spontaneous and precipitated withdrawal after chronic intragastric administration of γ-hydroxybutyrate (GHB) in baboons. Psychopharmacology 179:678–687.

Wong CG, Gibson KM, Snead OC 3rd (2004) From the street to the brain: neurobiology of the recreational drug γ-hydroxybutyric acid. Trends Pharmacol Sci 25:29–34.

Zhang X, Ju G, Le Gal La Salle G (1991) Fos expression in GHB-induced generalized absence epilepsy in the thalamus of the rat. Neuroreport 2:469–472.

(Accepted 28 March 2006)
(Available online 3 May 2006)