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Brain Research 997 (2004) 62–66
BRAIN RESEARCH
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Short communication

Gamma-hydroxybutyrate and ethanol depress spontaneous excitatory postsynaptic currents in dopaminergic neurons of the substantia nigra

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Accepted 7 October 2003

Abstract

Gamma-hydroxybutyrate (GHB) has been shown to have therapeutical properties in various psychiatric disorders, especially in alcohol abuse, and to mimic different actions of ethanol at the cellular and system level. Using whole-cell patch-clamp recordings on brain slices of 21- to 25-day-old rats, the present study investigated the effects of GHB and ethanol on spontaneous excitatory postsynaptic currents (sEPSCs) in dopaminergic neurons of the substantia nigra pars compacta (SNc). sEPSCs are an index of glutamate release from the excitatory input to dopamine cells, which play a key role in different reward-related behaviors. We found that GHB and ethanol depressed both the frequency and the amplitude of sEPSCs. These effects were $GABA_B$-independent and the GHB-induced depression was blocked by the GHB receptor antagonist 6,7,8,9-tetrahydro-5[*H*]benzocyclohepte-5-ol-4-ylidene acetic acid (NCS-382), pointing to a specific effect of this drug. The effects of ethanol were not affected by NCS-382. This study indicates that GHB and ethanol share the effect of reducing the efficacy of excitatory glutamatergic neurotransmission in the SNc by acting through different mechanisms.

Keywords: Gamma-hydroxybutyric acid; Ethanol; NCS-382; Dopaminergic neuron; Substantia nigra pars compacta; Spontaneous excitatory postsynaptic current

Gamma-hydroxybutyric acid (GHB) is a short-chain fatty acid bearing an alcoholic moiety. It was reported by Bessman and Fishbein [3] to be a natural constituent of the brain. The regional distribution of GHB is uneven, the greatest amount being in the substantia nigra, hippocampus and thalamus. At cellular level, GHB is located mainly in the cytosol, where it is synthesized from succinic semialdehyde through the intervention of succinic semialdehyde reductase [21]. Ethanol is, worldwide, the most consumed drug of addiction. It affects virtually all body organs, but it is abused for its effects on the central nervous system. GHB has been featured as an effective agent in the pharmacotherapy of alcohol dependence owing to its ability to reduce alcohol craving and consumption, promote abstinence and alleviate the symptoms of alcohol withdrawal syndrome [1,12].

The substantia nigra is located in the midbrain and contains the cell bodies of dopaminergic neurons. Dopaminergic neurons give rise to diffuse mesolimbic and nigrostriatal projections, which play a major role in the actions of many drugs of abuse, including ethanol self-administration. Dopaminergic projections are involved in addiction by controlling reward effects of psychoactive substances and by generating compulsive drug seeking behaviors [16,20]. Excitatory synaptic input to the substantia nigra mediated by glutamate is a key component of the regulation of dopamine cells, which express NMDA and non-NMDA glutamate receptors that contribute to the generation of synaptic responses [5,19]. The glutamatergic afferents arise mainly from the medial prefrontal cortex and from the subthalamic nucleus [11]. One role of the glutamatergic innervation to the substantia nigra is to mediate a switch from pacemaker-like firing in dopamine cells to burst-firing [13,30].

GHB and ethanol, sharing many neuropharmacological properties [14], have been shown to reduce excitatory neurotransmission in different brain systems [2,26,27]. How-

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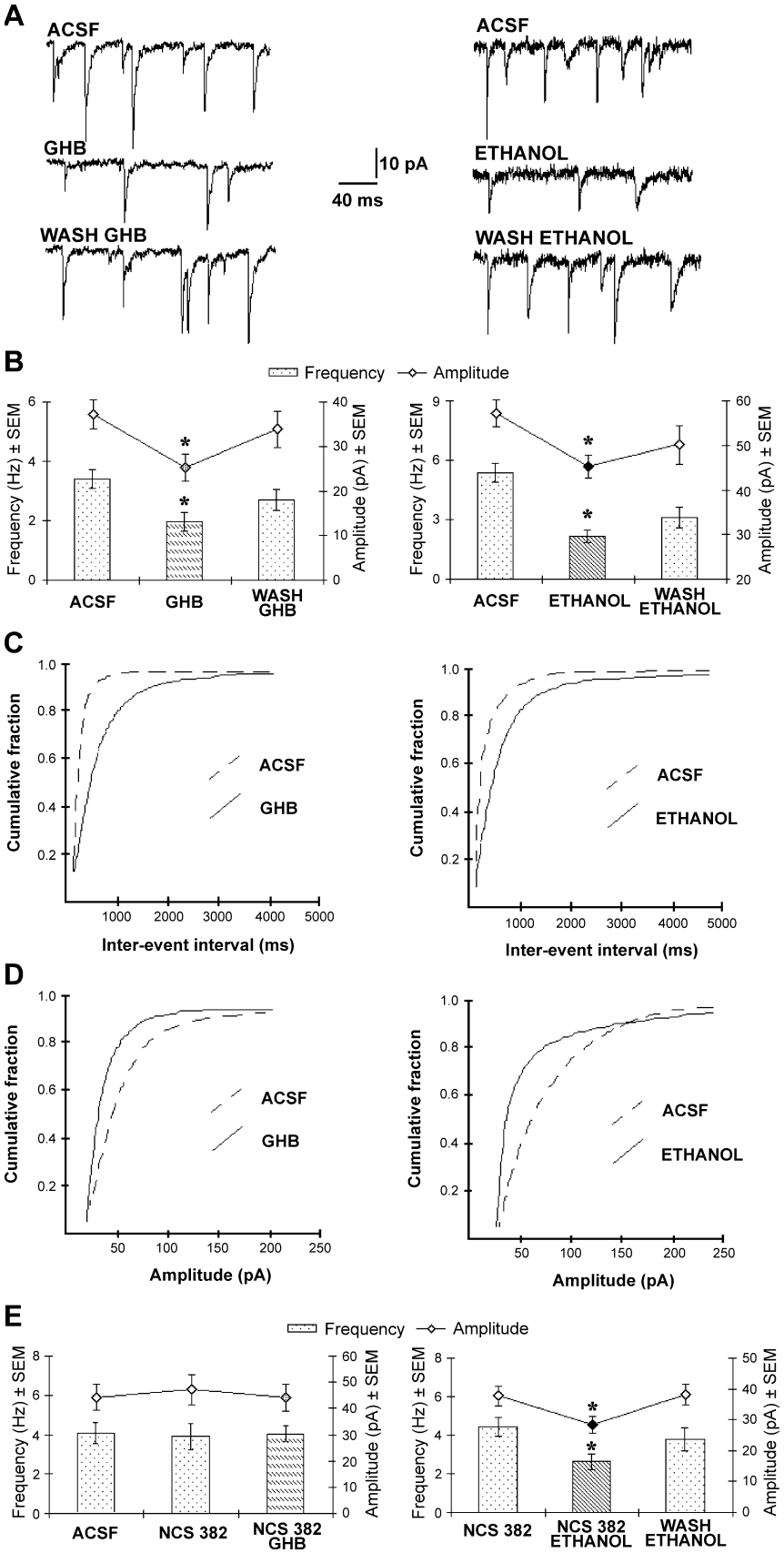
doi:10.1016/j.brainres.2003.10.046


A
ACSF
GHB
WASH GHB
10 pA
40 ms
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Frequency
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Frequency (Hz) ± SEM
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ACSF
GHB
WASH GHB
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Cumulative fraction
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Inter-event interval (ms)
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ETHANOL
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Frequency
Amplitude
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NCS 382
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ever, the effects of these drugs on spontaneous excitatory transmission of midbrain dopaminergic neurons have not yet been investigated and could play an important role in alcohol pharmacotherapy of GHB. The goal of the present study was to determine the acute actions of GHB and ethanol on spontaneous excitatory synaptic transmission in the substantia nigra pars compacta (SNc) as revealed by whole-cell patch-clamp recordings of spontaneous excitatory postsynaptic currents (sEPSCs) from dopaminergic neurons. Dopaminergic neurons of the SNc represent the origin of the nigrostriatal dopaminergic system and are directly involved in addiction, generating compulsive drug intake [23].

Experiments were performed on horizontal slices of the rat ventral midbrain containing the substantia nigra pars compacta, obtained as described previously [24]. Briefly, male Wistar rats (P21–P25, 60–80 g) were anesthetized with intraperitoneal ketamine injection and decapitated. The brain was rapidly removed from the skull and horizontal slices (250 μm thick) were cut in 8–12 °C artificial cerebrospinal fluid (ACSF) solution using a vibratome. After 1 h of incubation in 34 °C ACSF, containing in mM 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 1.2 NaH_2PO_4, 2.4 $CaCl_2$, 10 glucose, 19 $NaHCO_3$ and gassed with 95% O2–5% CO_2, a single slice was transferred to a recording chamber. Here, the slice was completely submerged in continuously flowing (2.5 ml/min) ACSF (33–34 °C, pH 7.4) and immobilized with a platinum grid. 4-AP (50 μM) and 3-*N*[1-(*s*)-(3,4-dichlorophenyl) ethyl] amino-2(*s*)-hydroxypropyl-*p*-benzyl-phosphinic acid (CGP-55845A; 1 μM) were added to the bath solution, respectively, to enhance the frequency of spontaneous excitatory postsynaptic currents and to prevent aspecific GHB-driven $GABA_B$ receptor activation [32]. The chamber was mounted on the stage of an upright microscope Axioskop (Carl Zeiss, Oberkochen, Germany) equipped for infrared video microscopy (Hamamatsu, Hamamatsu City, Japan).

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the whole cell configuration of the patch-clamp technique (voltage-clamp mode) with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA), at a holding potential of −60 mV. Patch electrodes, pulled with a PP-83 Narishige puller (Tokyo, Japan) using 1.5-mm borosilicate glass, had a resistance of 3–6 MΩ when filled with an intracellular solution containing (in mM) 130 K-Gluconate, 2 $MgCl_2$, 1 $CaCl_2$, 1 NaCl, 11 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Na_3-GTP; pH was adjusted to 7.3 with KOH. Individual neurons within the SNc were visualized by infrared video imaging and approached by applying positive pressure in the electrode. Then they were recognized as dopaminergic on the basis of the presence of a large hyperpolarization-induced inward current, typical of these cells [18,24]. Series resistance and whole-cell capacitance were monitored continuously during the experiment and recordings were discarded if series resistance changed by more than 20% from control conditions. Drugs were dissolved in ACSF and applied in the bath by changing the superfusion solution to one that differed only in its content of drug(s). The ratio of flow rate to bath volume ensured complete exchange within 1 min. Drugs used were: gamma-hydroxybutyrate (GHB), CGP 55845A and 6,7,8,9-tetrahydro-5[*H*]benzocyclohepte-5-ol-4-ylidene acetic acid (NCS-382) from Laboratorio Farmaceutico CT (San Remo, Italy); 4-aminopyridine (4-AP), ethanol and bicuculline from Sigma. Recordings were digitized online with the p-Clamp 7 software (Axon Instruments) using a Digidata 1200 acquisition board (Axon Instruments). Spontaneous events were analyzed with the MiniAnalysis software (Synaptosoft, Decatur, GA). The data were presented as mean ± S.E.M. Statistical differences were determined by paired analysis of variance test (ANOVA) and Kolmogorov–Smirnov test at a significance level of 0.05.

We recorded from 25 dopaminergic neurons within the SNc. Dopaminergic cells were identified by their prominent hyperpolarization-activated inward current [18,24]. Neurons had a mean input resistance of 106 ± 18 MΩ, which was not statistically affected by 600 μM GHB (105 ± 32 MΩ, $p = 0.66$), nor by 66 mM ethanol (109 ± 35 MΩ, $p = 0.76$). sEPSCs were resistant to bicuculline (30 μM, $n = 2$) and appeared as rapidly rising inward currents with a mean frequency of 3.41 ± 0.32 Hz and mean amplitudes of 37.2 ± 3.2 pA. Perfusion with GHB (600 μM) reduced the sEPSCs frequency to 1.96 ± 0.31 Hz (−42.5 ± 9.1%; $F(1,5) = 66.1$, $p < 0.01$) and the mean amplitudes to 25.21 ± 3 pA (−32.3 ± 8.1%; $F(1,5) = 39.8$, $p < 0.01$; $n = 6$). After 10 min of GHB perfusion, washout showed a recovery of the sEPSCs frequency to 2.71 ± 0.36 Hz and a recovery of the sEPSCs amplitude to 33.9 ± 4.1 pA. Inter-event interval and amplitude distributions were significantly different between control and GHB ($p < 0.01$, KS test) in four of five cells tested (Fig. 1A–D, left panels). To characterize the receptors involved in GHB-induced effects, we repeated the experiments in the presence of the GHB antagonist NCS-382 in four neurons. As shown in Fig. 1E (left panel), NCS-382 (500 μM) applied alone for 10 min

Fig. 1. Effects of GHB (600 μM) and ethanol (66 mM) on sEPSCs. (A) Left panel: recordings of sEPSCs in control, during GHB perfusion and upon washout of GHB in a representative neuron. Right panel: recordings of sEPSCs in control, during ethanol perfusion and upon washout of ethanol in a representative neuron. (B) Summary of the mean effect of GHB (left panel, $n = 6$) and ethanol (right panel, $n = 8$) on the frequency and amplitude of sEPSCs. Significant reductions (*) were observed for both drugs in both frequency and amplitude. (C) Cumulative inter-event interval distribution of a representative cell, revealing a significant increase in the inter-event interval (i.e., decreased frequency) during GHB (left panel) and ethanol (right panel) application. (D) Cumulative amplitude distribution obtained from the same cells reveals a significant decrease in sEPSCs' amplitude during GHB (left panel) and ethanol (right panel) application. (E) Left panel: NCS-382 (500 μM), an antagonist of GHB receptor, blocks the effects of GHB on sEPSCs. Application of NCS-382 alone showed no effects, but the effects of GHB on both frequency and amplitude were blocked ($n = 4$). Right panel: NCS-382 was not able to block the effects of ethanol on both sEPSCs' frequency and amplitude ($n = 7$).

had no significant effects on the frequency (from 4.02 ± 0.51 to 3.95 ± 0.66 Hz) and amplitude (from 44.3 ± 5.1 to 47.1 ± 5.9 pA) of the sEPSCs. However, NCS382 (500 μM) was able to significantly block the effects of GHB (600 μM) on the frequency (+1.7 ± 16.4%, $p>0.05$) and amplitude (−6.3 ± 13.5%; $p>0.05$, $n=4$) of the sEPSCs.

Bath application of ethanol (66 mM) reduced sEPSCs frequency from 5.36 ± 0.43 to 2.33 ± 0.4 Hz (−56.5 ± 7.5%; $F(1,7)=45.5$, $p<0.01$) and sEPSCs mean amplitudes from 56.2 ± 2.3 to 44.1 ± 2.1 pA (−21.6 ± 3.7%; $F(1,7)=31.2$, $p<0.01$, $n=8$). After 10 min of ethanol perfusion, washout showed a recovery of the sEPSCs frequency to 3.06 ± 0.52 Hz and a recovery of the sEPSCs amplitude to 49.5 ± 2.9 pA. Inter-event interval and amplitude distributions were significantly different between control and ethanol ($p<0.01$, KS test) in all cells tested (Fig. 1A–D, right panels). We repeated the experiments in the presence of NCS-382 to test whether this drug was able to block also the depression effects of ethanol. As shown in Fig. 1E (right panel), in the presence of NCS-382 bath application of ethanol still reduced sEPSCs frequency (from 4.41 ± 0.25 to 2.63 ± 0.41 Hz; −40.36 ± 4.12%, $p<0.05$) and amplitude (from 37.6 ± 2.9 to 28.3 ± 2.7 pA; −24.74 ± 2.3%, $n=7$).

Our results show that GHB and ethanol reversibly reduce both frequency and amplitude of sEPSCs in dopaminergic neurons of the SNc. These results are in line with previous studies, showing that GHB and ethanol exert depressant effects on excitatory transmission in diverse brain areas [2,4,17,27]. The concentrations of GHB and ethanol used in this study (600 μM and 66 mM, respectively) are in the range of those measured in the rat brain after systemic administration of pharmacologically effective doses of GHB (200–400 mg/kg) that reduced voluntary alcohol intake [9] and of those measured in the rat brain (1–5 g/kg) during expression of addiction effects of ethanol [15,31]. Moreover, various in vitro studies have previously shown that maximal acute effects of GHB and ethanol occur at these concentrations [2,6,26,27]. Differently from previous reports [28,32], we never observed changes in the holding current or on the cell input resistance. This difference is probably due to the continuous presence of CGP 55845A in the perfusing medium of our experiments, in order to prevent any effect on $GABA_B$ receptors. Indeed, both drugs tested in this study are known to activate presynaptic $GABA_B$ receptors [29,32]. However, in contrast with ethanol whose mechanisms of action are not specific and very spread [8], the effect of GHB observed here has to be ascribed to specific depressing mechanisms, since its effect on sEPSCs frequency and amplitude was prevented by NCS-382, a GHB receptor antagonist [22]. The evidence that the presence of a specific antagonist of GHB receptor in the perfusing medium blocked the effects of GHB suggests that GHB depressant effect is mediated by the activation of its own receptor [7].

The inhibitory effects of GHB and ethanol on spontaneous glutamatergic neurotransmission revealed by this study are expected to have substantial influences on the activity of dopaminergic neurons of the ventral midbrain, and consequently on a wide range of behaviors related to ethanol abuse and GHB pharmacotherapy of alcohol dependence. Furthermore, the similarity of GHB and ethanol effects here reported provides support to the idea that GHB might prevent ethanol withdrawal syndrome in both laboratory animal and in man by mimicking ethanol actions in the central nervous system [10]. However, some mechanisms behind the interactions between GHB and ethanol, such as the abuse of GHB and its co-abuse with ethanol, wait to be further explored [25].

References

[1] G. Addolorato, F. Caputo, E. Capristo, G.F. Stefanini, G. Gasbarrini, Gamma-hydroxybutyric acid: efficacy, potential abuse, and dependence in the treatment of alcohol addiction, Alcohol 20 (2000) 217–222.

[2] F. Berton, A. Brancucci, F. Beghè, M. Cammalleri, A. Demuro, W. Francesconi, G.L. Gessa, Gamma-hydroxybutyrate inhibits excitatory postsynaptic potentials in rat hippocampal slices, Eur. J. Pharmacol. 380 (1999) 109–116.

[3] S.P. Bessman, W.N. Fishbein, Gamma-hydroxybutyrate, a normal brain metabolite, Nature 200 (1963) 1207–1208.

[4] F.E. Bloom, G.R. Siggins, Electrophysiological action of ethanol at the cellular level, Alcohol 4 (1987) 331–337.

[5] A. Bonci, R.C. Malenka, Properties and plasticity of excitatory synapses on dopaminergic and GABAergic cells in the ventral tegmental area, J. Neurosci. 19 (1999) 3723–3730.

[6] M. Cammalleri, A. Brancucci, F. Berton, A. Locche, G.L. Gessa, W. Francesconi, Gamma-hydroxybutyric acid reduces GABA-mediated inhibitory postsynaptic potentials in the CA1 region of hippocampus, Neuropsychopharmacology 27 (2002) 960–969.

[7] M.P. Castelli, I. Mocci, M. Pistis, M. Peis, D. Berta, A. Gelain, G.L. Gessa, G. Cignarella, Stereoselectivity of NCS-382 binding to gamma-hydroxybutyrate receptor in the rat brain, Eur. J. Pharmacol. 446 (2002) 1–5.

[8] F. Fadda, Z.L. Rossetti, Chronic ethanol consumption: from neuroadaptation to neurodegeneration, Prog. Neurobiol. 56 (1998) 385–431.

[9] F. Fadda, E. Mosca, G. Colombo, G.L. Gessa, Gamma-hydroxybutyric acid suppresses ethanol consumption in alcohol-preferring rats, Psychopharmacology 96 (1988) 107.

[10] F. Fadda, G. Colombo, E. Mosca, G.L. Gessa, Suppression by gamma-hydroxybutyric acid of ethanol withdrawal syndrome in rats, Alcohol Alcohol. 24 (1989) 447–451.

[11] J.H. Fallon, S.E. Loughlin, Substantia nigra, in: G. Paxinos (Ed.), The Rat Nervous System, Academic Press, New York, 1995, pp. 215–237.

[12] L. Gallimberti, M.R. Spella, C.A. Soncini, G.L. Gessa, Gamma-hydroxybutyric acid in the treatment of alcohol and heroin dependence, Alcohol 20 (2000) 257–262.

[13] R.F. Gariano, P.M. Groves, Burst firing induced in midbrain dopamine neurons by stimulation of the medial prefrontal and anterior cingulated cortices, Brain Res. 462 (1988) 194–198.

[14] G.L. Gessa, R. Agabio, M.A.M. Carai, C. Lobina, M. Pani, R. Reali, G. Colombo, Mechanism of the antialcohol effect of gamma-hydroxybutyric acid, Alcohol 20 (2000) 271–276.

[15] C.W. Hodge, K.K. Mehmert, S.P. Kelley, T. McMahon, A. Haywood, M.F. Olive, D. Wang, A.M. Sanchez-Perez, R.O. Messing, Supersensitivity to allosteric $GABA_A$ receptor modulators and alcohol in mice lacking PKCe, Nat. Neurosci. 2 (1999) 997–1002.

[16] S.E. Hyman, R.C. Malenka, Addiction and the brain: the neurobiology of compulsion and its persistence, Nat. Rev., Neurosci. 2 (2001) 695–703.
[17] K. Jensen, I. Mody, GHB depresses fast excitatory and inhibitory synaptic transmission via $GABA_B$ receptors in mouse neocortical neurons, Cereb. Cortex 11 (2001) 424–429.
[18] S.W. Johnson, R.A. North, Two types of neurons in the rat ventral tegmental area and their synaptic inputs, J. Physiol. (London) 450 (1992) 455–468.
[19] P.W. Kalivas, Neurotransmitter regulation of dopamine neurons in the ventral tegmental area, Brain Res. Rev. 18 (1993) 75–113.
[20] P.W. Kalivas, J. Steward, Dopamine transmission in the initiation and expression of drug- and stress-induced sensitisation of motor activity, Brain Res. Rev. 16 (1991) 223–244.
[21] M. Maitre, The gamma-hydroxybutyrate signalling system in the brain: organization and functional implications, Prog. Neurobiol. 51 (1997) 337–361.
[22] M. Maitre, V. Hechler, P. Vayer, S. Gobaille, C.D. Cash, M. Schmitt, J.J. Bourguignon, A specific gamma-hydroxybutyrate receptor ligand possesses both antagonistic and anticonvulsant properties, J. Pharmacol. Exp. Ther. 255 (1990) 657–663.
[23] K. McFarland, P.W. Kalivas, The circuitry mediating cocaine-induced reinstatement of drug-seeking behavior, J. Neurosci. 21 (2001) 8655–8663.
[24] N.B. Mercuri, A. Bonci, P. Calabresi, F. Stratta, G. Bernardi, Properties of the hyperpolarization-activated cation current Ih in rat midbrain dopaminergic neurons, Eur. J. Neurosci. 7 (1995) 462–469.
[25] K.L. Nicholson, R.L. Balster, GHB: a new and novel drug of abuse, Drug Alcohol Depend. 63 (2001) 1–22.
[26] Z. Nie, X. Yuan, S.G. Madamba, G.R. Siggins, Ethanol decreases glutamatergic synaptic transmission in rat nucleus accumbens in vitro: naloxone reversal, J. Pharmacol. Exp. Ther. 266 (1993) 1705–1712.
[27] Z. Nie, S.G. Madamba, G.R. Siggins, Ethanol inhibits glutamatergic neurotransmission in nucleus accumbens neurons by multiple mechanisms, J. Pharmacol. Exp. Ther. 271 (1994) 1566–1573.
[28] H.R. Olpe, W.P. Koella, Inhibition of nigral and neocortical cells by gamma-hydroxybutyrate: a microiontophoretic investigation, Eur. J. Pharmacol. 53 (1979) 359–364.
[29] S.C. Steffensen, Z. Nie, J.R. Criado, G.R. Siggins, Ethanol inhibition of *N*-methyl-D-aspartate responses involves presynaptic gamma-aminobutyric acid (B) receptors, J. Pharmacol. Exp. Ther. 294 (2000) 637–647.
[30] T.H. Svensson, C.S. Tung, Local coding of prefrontal cortex induces pacemaker-like firing of dopamine neurons in rat ventral tegmental area in vivo, Acta Physiol. Scand. 136 (1989) 135–136.
[31] T.E. Thiele, B. Willias, J. Stadler, J.G. Reynolds, I.L. Bernestein, G.S. McKnight, High ethanol consumption and low sensitivity to ethanol-induced sedation in protein kinase A-mutant mice, J. Neurosci. 20 (2000) 1–6.
[32] X. Xie, T.G. Smart, Gamma-hydroxybutyrate hyperpolarizes hippocampal neurons by activating $GABA_B$ receptors, Eur. J. Pharmacol. 212 (1992) 291–294.