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Additive inhibition of human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptors by mixtures of commonly used drugs of abuse

Laura Hondebrink [a,1,*], Sijie Tan [b,1,2], Elise Hermans [b], Regina G.D.M. van Kleef [b], Jan Meulenbelt [a,b,c], Remco H.S. Westerink [b]

[a] National Poisons Information Center (NVIC), University Medical Center Utrecht, P.O. Box 85500, NL-3508 GA Utrecht, The Netherlands
[b] Neurotoxicology Research Group, Division Toxicology, Institute for Risk Assessment Sciences (IRAS), Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.177, NL-3508 Utrecht, The Netherlands
[c] Department of Intensive Care Medicine, University Medical Center Utrecht, P.O. Box 85500, NL-3508 GA Utrecht, The Netherlands

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ABSTRACT

Yearly, exposure to drugs of abuse results in ~1 million emergency department visits in the US. In ~50% of the visits, stimulant drugs like cocaine and amphetamine-like substances (e.g. 3,4-methylenedioxymethamphetamine (MDMA, the main active ingredient of ecstasy)) are involved, whereas in ~60% multiple drugs are involved. These drugs induce higher dopamine and serotonin levels resulting in drug-induced toxicity. Since GABA receptors (GABA-R) provide the main inhibitory input on dopaminergic and serotonergic neurons, drug-induced inhibition of GABA-R could contribute to higher neurotransmitter levels and thus toxicity.

We therefore investigated the effects of combinations of commonly abused stimulant drugs (cocaine, MDMA, 3,4-methylenedioxyamphetamine (MDA) and *meta*-chlorophenylpiperazine (*m*CPP)) on the function of the human $\alpha_1\beta_2\gamma_2$ $GABA_A$ receptor ($hGABA_A$-R), expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique.

These drugs concentration-dependently inhibited the GABA-evoked current (*m*CPP > cocaine > MDMA > MDA). Most drug combinations decreased the GABA-evoked current stronger than the single drug. Additivity was observed during combined exposure to low concentrations of cocaine and *m*CPP as well as during combined exposure to MDA with cocaine or *m*CPP. However, combinations containing MDMA mainly resulted in sub-additivity or no additivity.

At drug concentrations relevant for clinical toxicology, co-exposure to ≥2 drugs can decrease the GABA-evoked current in an additive manner. Thus, in patients exposed to multiple drugs, inhibitory GABA-ergic input is reduced more prominently, likely resulting in higher brain dopamine levels. As this will increase the risk for drug-induced toxicity, treatment of drug-intoxicated patients with drugs that enhance GABA-ergic input should be further optimized.

1. Introduction

Drugs of abuse can be classified into several groups, i.e., cannabinoids (e.g. marihuana), opioids (e.g. heroin), stimulants (e.g. cocaine and (meth)amphetamine), club drugs (e.g. ecstasy tablets with the active substance 3,4-methylenedioxymethamphetamine (MDMA)), dissociative drugs (e.g. ketamine) and hallucinogens (e.g. LSD and psilocybin) (NIH & NIDA, 2011). In

Abbreviations: 5-HT, serotonin; cDNA, complementary DNA; DA, dopamine; DAT, dopamine transporter; EC_{20}, effective concentration that evokes 20% of the maximum response; ED, emergency department; GABA, γ-aminobutyric acid; $GABA_A$-R, $GABA_A$ receptor; $hGABA_A$-R, human $GABA_A$ receptor; *m*CPP, *meta*-chlorophenylpiperazine; MDA, 3,4-methylenedioxyamphetamine; MDMA, 3,4-methylenedioxymethamphetamine; SERT, serotonin reuptake transporter.

* Corresponding author at: University Medical Center Utrecht, Division of Anesthesiology, Intensive Care and Emergency Medicine, National Poisons Information Center (NVIC), P.O. Box 85500, 3508 GA Utrecht, The Netherlands. Tel.: +31 88 755 9542.
E-mail addresses: L.Hondebrink@umcutrecht.nl (L. Hondebrink), sijietan@gmail.com (S. Tan), E.J.P.hermans@uu.nl (E. Hermans), G.vankleef@uu.nl (Regina G.D.M. van Kleef), J.Meulenbelt-1@umcutrecht.nl (J. Meulenbelt), R.Westerink@uu.nl (Remco H.S. Westerink).

[1] These authors contributed equally to this work.
[2] Present address: Department of Histology and Embryology, School of Medicine, University of South China, 421001 Hengyang, PR China.

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Europe, the highest prevalence is observed for the use of cannabinoids. However, significant prevalences are also observed for the use of stimulants and club drugs, with lifetime prevalences for the use of cocaine and amphetamines (MDMA, (meth)amphetamine) of 5% and 4% respectively (age group 15–64, EMCDDA, 2011).

Although cocaine and (meth)amphetamine have a much lower user prevalence compared to cannabinoids, they were involved in ~50% of the one million drug-related emergency department (ED) visits in the US (40% cocaine and 10% (meth)amphetamine), compared to 40% of the visits due to cannabinoids. Of the ED visits involving an illicit drug, a quarter of all patients are admitted to the hospital (SAMHSA, 2011), where they are often treated with benzodiazepines to control blood pressure (in agitated patients), neurological complications such as seizures and to control psychiatric complications that hamper treatment of the patient (Gist et al., 2011; McCormack and Buckley, 2006). Benzodiazepines modulate the inhibitory γ-aminobutyric acid$_A$ receptor (GABA$_A$-R), thereby increasing GABA-ergic input. Although no human clinical trials have been published to show the effectiveness of benzodiazepine treatment during a drug intoxication, animal data has shown a 50% reduction in mortality due to cocaine poisoning following benzodiazepine treatment (Heard et al., 2011). Moreover, clinical practice has shown that administration of benzodiazepines improves drug-induced symptoms in humans. Considering the beneficial effects of benzodiazepines in treatment of drug intoxications, it is plausible that drugs of abuse actually decrease GABA-ergic input.

Many stimulants and club drugs are known for their ability to acutely increase neurotransmitter levels, mainly dopamine (DA) and serotonin (5-HT). This is primarily due to inhibition and/or reversal of neurotransmitter reuptake transporters, like the DA membrane transporter (DAT) and the 5-HT membrane transporter (SERT). Redistribution of DA and 5-HT from synaptic vesicles into the cytosol could also contribute to this mode of action. Additionally, some drugs have agonistic effects on neurotransmitter receptors, e.g., MDMA can activate 5-HT$_2$ receptors. Furthermore, inhibitory effects were observed on acetylcholine receptors as well as on GABA-Rs (Hondebrink et al., 2011a, 2012). Pharmacological blockage of specific receptors inhibited MDMA-induced DA and 5-HT release, indicating receptor activation as an important additional mechanism of action (for reviews see Sulzer et al., 2005; Capela et al., 2009; Rietjens et al., 2012). Dopaminergic and serotonergic neurons receive multiple inputs from other neurotransmitter systems, including the GABA-ergic system that provides the major inhibitory input in the brain. Activation of GABA-Rs can decrease dopaminergic and serotonergic neurotransmission, while inhibition of GABA-R can increase it (D'Hulst et al., 2009; Kiyatkin and Rebec, 1998; McKernan and Whiting, 1996). Although the GABA-ergic system has received little attention in the field of drugs of abuse, (inhibitory) interactions between drugs of abuse and GABA-Rs could thus increase DA and 5-HT release. The fact that intoxicated patients are often successfully treated with benzodiazepines (GABA$_A$-agonists) further supports this hypothesis.

The $\alpha_1\beta_2\gamma_2$ GABA$_A$-R subtype combination is the most abundant subtype in many brain areas, such as hippocampus and cortex (McKernan and Whiting, 1996). Importantly, GABA-ergic signaling in dopaminergic brain areas, like the ventral tegmental area and the substantia nigra compacta, is also mainly mediated by the $\alpha_1\beta_2\gamma_2$ GABA$_A$-R subtype (Petri et al., 2002). Our previous study showed that several widely used drugs of abuse can modulate the function of human $\alpha_1\beta_2\gamma_2$ GABA$_A$-R when co-applied with the natural agonist GABA (Hondebrink et al., 2011a). However, in ~60% of drug-related ED visits, multiple drugs are involved (SAMHSA, 2011). Furthermore, the impurity of drugs of abuse is well established; ecstasy tablets contain different amounts of MDMA and regularly also other psychoactive substances, e.g., *meta*-chlorophenylpiperazine (*m*CPP) and MDMA-like substances such as 3,4-methylenedioxyamphetamine (MDA) (Parrott, 2004; van Laar et al., 2010). Therefore, we investigated the effects of mixtures of several popular drugs of abuse (MDMA, its primary metabolite MDA, *m*CPP and cocaine) on the function of GABA$_A$-Rs, expressed in *Xenopus* oocytes, using the two-electrode voltage-clamp technique.

2. Materials and methods

2.1. Animals

Xenopus laevis oocytes were used to investigate drug-induced effects on GABA$_A$-R function. Oocytes efficiently translate injected complementary (cDNA) into RNA and RNA into protein. Neurophysiological studies and studies using labeled proteins have shown that the expressed proteins are functional and transported to areas where they normally occur (Brown, 2004). This model thus enables direct measurement of receptor function. Consequently, the *X. laevis* oocyte is widely used for the expression of heterologous proteins and was considered the most suitable model for the present study.

Experiments were approved by the Ethical Committee for Animal Experiments of Utrecht University and conducted in accordance with Dutch law and the European Community directives regulating animal research (2010/63/EU). All efforts were made to minimize the number of animals used and their suffering. Mature female *X. laevis* frogs (provided by Dr. Scheenen, Radboud University Nijmegen, The Netherlands) were kept in standard aquaria (0.5 m × 0.4 m × 1 m; 1–10/aquarium) with copper-free water (pH 6.5, 21–23 °C) and a 12 h light/dark cycle. Animals were fed earthworms three times a week (Hagens, Nijkerkerveen, The Netherlands).

2.2. Chemicals

DL-3,4-Methylenedioxymethamphetamine (MDMA), DL-3,4-methylenedioxyamphetamine (MDA) and 1-(3-chlorophenyl)-piperazine (*m*CPP) were obtained from Duchefa (Haarlem, The Netherlands). Cocaine was obtained from Spruyt Hillen (IJsselstein, The Netherlands). Stock-solutions (1 M) were prepared in saline and stored at 4 °C for no more than 2 weeks. $CaCl_2$, $Ca(NO_3)_2$, KCl, $MgCl_2$, $MgSO_4$, $NaHCO_3$, NaOH and HEPES were purchased from Merck (Darmstadt, Germany). All other chemicals, unless otherwise noted, were obtained from Sigma–Aldrich (Zwijndrecht, The Netherlands).

2.3. Expression of $\alpha_1\beta_2\gamma_2$ GABA$_A$ receptors in X. laevis oocytes

All procedures have been described previously (Fernandes et al., 2010; Hendriks et al., 2010; Hondebrink et al., 2011a). Briefly, female *X. laevis* were anesthetized by submersion in 0.1% MS-222, and ovarian lobes were surgically removed. Oocytes were incubated with collagenase type I (1.5 mg/ml Ca^{2+}-free Barth's solution) for 2 h at room temperature. cDNA coding for the human α_1, β_2 and γ_2 subunits of human GABA$_A$ receptors (Origene, Rockville, USA) was dissolved in distilled water at a 1:1:1 molar ratio and injected (23 nl/oocyte, ~1 ng of each subunit) into the nuclei of stage V or VI oocytes using a Nanoject Automatic Oocyte Injector (Drummond, Broomall, PA). The obtained oocytes were incubated for 2–5 days at 21 °C in modified Barth's solution containing (in mM) 0.41 $CaCl_2$, 0.3 $Ca(NO_3)_2$, 15 HEPES, 1 KCl, 0.82 $MgSO_4$, 88 NaCl, 2.4 $NaHCO_3$ and 50 μg/ml neomycin (pH 7.6 with NaOH) to allow for receptor expression.