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A STRESS STEROID TRIGGERS ANXIETY VIA INCREASED EXPRESSION OF α4βδ $GABA_A$ RECEPTORS IN METHAMPHETAMINE DEPENDENCE

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Abstract—Methamphetamine (METH) is an addictive stimulant drug. In addition to drug craving and lethargy, METH withdrawal is associated with stress-triggered anxiety. However, the cellular basis for this stress-triggered anxiety is not understood. The present results suggest that during METH withdrawal (24 h) following chronic exposure (3 mg/kg, i.p. for 3–5 weeks) of adult, male mice, the effect of one neurosteroid released by stress, 3α,5α-THP (3α-OH-5α-pregnan-20-one), and its 3α,5β isomer reverse to trigger anxiety assessed by the acoustic startle response (ASR), in contrast to their usual anti-anxiety effects. This novel effect of 3α,5β-THP was due to increased (three-fold) hippocampal expression of α4βδ $GABA_A$ receptors (GABARs) during METH withdrawal (24 h–4 weeks) because anxiogenic effects of 3α,5β-THP were not seen in α4−/− mice. 3α,5β-THP reduces current at these receptors when it is hyperpolarizing, as observed during METH withdrawal. As a result, 3α,5β-THP (30 nM) increased neuronal excitability, assessed with current clamp and cell-attached recordings in CA1 hippocampus, one CNS site which regulates anxiety. α4βδ GABARs were first increased 1 h after METH exposure and recovered 6 weeks after METH withdrawal. Similar increases in α4βδ GABARs and anxiogenic effects of 3α,5β-THP were noted in rats during METH withdrawal (24 h). In contrast, the ASR was increased by chronic METH treatment in the absence of 3α,5β-THP administration due to its stimulant effect. Although α4βδ GABARs were increased by chronic METH treatment, the GABAergic current recorded from hippocampal neurons at this time was a depolarizing, shunting inhibition, which was potentiated by 3α,5β-THP. This steroid reduced neuronal excitability and anxiety during chronic METH treatment, consistent with its typical effect. Flumazenil (10 mg/kg, i.p., 3×) reduced α4βδ expression and prevented the anxiogenic effect of 3α,5β-THP after METH withdrawal. Our findings suggest a novel mechanism underlying stress-triggered anxiety after METH withdrawal mediated by α4βδ GABARs.

Key words: CA1 hippocampus, pregnanolone, allopregnanolone, stress, flumazenil, methamphetamine.

INTRODUCTION

Methamphetamine (METH) is an addictive, stimulant drug. Dependence on this drug is difficult to treat (Cretzmeyer et al., 2003; Shoptaw et al., 2009) because of the severity of the symptoms of METH withdrawal. In contrast to chronic METH exposure which is well-correlated with increased anxiety and hyperactivity (Barr et al., 2009) due to the stimulant actions of the drug, METH withdrawal is characterized by sedation and depression. However, stress can trigger paradoxical anxiety during METH withdrawal even though the stimulant effect of the drug is no longer present (London et al., 2004; Mancino et al., 2011). A variety of systems associated with the stress response, including norepinephrine, corticosterone and corticotropin-releasing factor (CRF), have been implicated in cocaine and ethanol withdrawal (Sarnyai et al., 1995; Basso et al., 1999; Zorrilla et al., 2001; Smith and Aston-Jones, 2008; Koob, 2009; Roberto et al., 2010). However, these systems have not been correlated with anxiety associated with withdrawal from amphetamines (Barr et al., 2010), suggesting alternative mechanisms. Although both METH and cocaine are stimulants, METH effects are distinct from cocaine due to its unique pharmacokinetics (Fowler et al., 2008). One CNS system not yet considered as a mediator of stress-triggered anxiety in METH withdrawal is the $GABA_A$ receptor (GABAR). This receptor is a likely candidate because it plays a pivotal role in generating anxiety and has a high degree of plasticity. Therefore the present study assessed the role of this system in METH dependence.

METH exerts stimulant actions in multiple brain regions, including the CA1 hippocampus (Hori et al., 2010), one CNS site which regulates anxiety (Bitran et al., 1999; Bannerman et al., 2004). METH increases glutamate release and depolarizes CA1 pyramidal cells when first administered (Yamamoto et al., 1999). These stimulant actions of the drug diminish during prolonged drug exposure (Yamamoto et al., 1999), suggesting that

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Abbreviations: 3α,5α-THP, 3α-OH-5α-pregnan-20-one or allopregnanolone; 3α,5β-THP, 3α-OH-5β-pregnan-20-one or pregnanolone; ANOVA, analysis of variance; ASR, acoustic startle response; CRF, corticotropin-releasing factor; EGTA, ethylene glycol tetraacetic acid; GABAR, $GABA_A$ receptor; GBX, gaboxadol or THIP (a GABA agonist); HEPES, hydroxyethyl piperazineethanesulfonic acid; I-threshold, The current threshold for triggering a spike; METH, methamphetamine; mIPSC, miniature inhibitory post-synaptic current; Rm, input resistance; sIPSC, spontaneous inhibitory post-synaptic current; TTX, tetrodotoxin.

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compensatory mechanisms occur to prevent neurotoxicity. Although compensatory mechanisms involving the glutamate and monoaminergic systems have been investigated after METH exposure (Yamamoto et al., 1999), compensatory regulation of the inhibitory GABAergic system in the CA1 hippocampus has not yet been studied in response to chronic METH exposure.

The GABAR is a pentameric membrane protein which gates a Cl^- current and is the primary source of inhibition in the brain (Olsen and Sieghart, 2009). These receptors can either localize sub-synaptically, where they generate a phasic current, or extrasynaptically (Wei et al., 2003), where they generate a tonic current (Bai et al., 2000; Stell and Mody, 2002) in response to ambient GABA. Of the many diverse sub-types, the extrasynaptic α4βδ GABAR normally has low expression in areas such as CA1 hippocampus (Pirker et al., 2000; Wei et al., 2003), an area in the limbic CNS important for generating mood (Bannerman et al., 2004). However, this receptor is highly regulatable, providing compensatory tonic inhibition across fluctuations in ovarian steroids as well as in response to increased neuronal excitability (Maguire et al., 2005; Shen et al., 2007; Mtchedlishvili et al., 2010), such as would occur with METH exposure. The α4βδ GABAR is also a sensitive target for 3α,5β-THP (3αOH-5β-pregnan-20-one or pregnanolone) and related neurosteroids, including 3α,5α-THP (3αOH-5α-pregnan-20-one or allopregnanolone) (Belelli et al., 2002; Brown et al., 2002; Bianchi and Macdonald, 2003). These THP isomers have nearly identical effects to increase GABA-gated current (Weir et al., 2004), and to reduce anxiety in adult rodents (Bitran et al., 1999; Rhodes and Frye, 2001; Toufexis et al., 2004).

3α,5α-THP is released during certain types of moderate stress in both rodents (Purdy et al., 1991; Mukai et al., 2008) and humans (Girdler et al., 2001; Droogleever Fortuyn et al., 2004). Studies conducted in humans have shown increases in circulating levels of this steroid during mental and/or social stress associated with performance (Girdler et al., 2001; Droogleever Fortuyn et al., 2004), which reflect stress states relevant for daily life. In contrast, stimulants such as cocaine do not have consistent effects in releasing 3α,5α-THP into the circulation (Grobin et al., 2005; Quinones-Jenab et al., 2008). There are also site-specific effects: recent studies show increased levels of 3α,5α-THP in striatum (Quinones-Jenab et al., 2008) but not in cortex (Grobin et al., 2005) or hippocampus (Quinones-Jenab et al., 2008) after cocaine administration to male rats at doses 5 to 10-fold greater than used in the present study. The source of stress-induced release of 3α,5α-THP includes the adrenal gland. However, the cellular machinery for 3α,5α-THP synthesis is also found in neurons such as CA1 pyramidal cells (Agis-Balboa et al., 2006), suggesting that this steroid (and its isomer, 3α,5β-THP) may be synthesized directly in the brain. This possibility is also suggested by studies showing stress-triggered 3α,5α-THP release in adrenalectomized animals (Purdy et al., 1991). Both THP isomers are metabolites of progesterone (Compagnone and Mellon, 2000), an ovarian hormone, and 3α,5α-THP is also increased on the proestrous stage of the estrous cycle in rodents (Palumbo et al., 1995) as well as before the onset of puberty (Fadalti et al., 1999; Shen et al., 2010b) and during pregnancy (Concas et al., 1998; Luisi et al., 2000) in both rodents and humans. Circulating levels of 3α,5α-THP and 3α,5β-THP are similar in men and (Porcu et al., 2010) and women (Havlikova et al., 2006; Porcu et al., 2009), and they are also altered in parallel in response to anti-depressant treatment (Girdler et al., 2012) suggesting that both isomers likely play a role in the stress response. In addition, neurosteroids have been shown to be neuroprotective (Rhodes et al., 2004) and anti-nociceptive (Charlet et al., 2008), suggesting they may have diverse functions in the CNS in addition to stress and reproductive function.

3α,5α-THP, 3α,5β-THP and related neurosteroids such as THDOC (3α,21-dihydroxy-5α-pregnan-20-one) are well known as potent positive modulators of the GABAR, where most definitive pharmacological studies have typically employed standard whole cell recording techniques to measure the effects on depolarizing, inward current (i.e., outward Cl^- flux). However, recent evidence suggests that neurosteroids have unique effects at α4βδ GABARs which are dependent upon the direction of Cl^- flux, such that they increase depolarizing current but decrease hyperpolarizing Cl^- current (Shen et al., 2007), an effect initially demonstrated in recombinant receptors. In dentate gyrus granule cells, which normally have a high level of α4βδ expression (Wei et al., 2003), the neurosteroid THDOC increases the tonic inhibitory current produced by α4βδ GABARs and reduces neuronal excitability (Stell et al., 2003; Chiang et al., 2012). While GABAergic current recorded from these cells has been shown to be depolarizing, it results in a shunting inhibition (Staley and Mody, 1992). Thus, the net effect of neurosteroids here would be to enhance this inhibition and decrease excitability, as has been demonstrated (Stell et al., 2003). However, in mature CA1 hippocampal pyramidal cells, GABAergic inhibition is hyperpolarizing (Lambert et al., 1991; Shen et al., 2007). Although, expression of α4βδ GABARs here is normally very low (Shen et al., 2010a), under conditions where expression of these receptors increases significantly, such as puberty, 3α,5β-THP produces a paradoxical excitatory effect (Shen et al., 2007). At the onset of puberty, 3α,5β-THP reduces the tonic inhibitory (hyperpolarizing) current, thereby increasing neuronal excitability *in vitro* and increasing anxiety (Shen et al., 2007). These effects are not observed in the δ−/− mouse, consistent with the polarity-dependent effect of the steroid observed in recombinant receptors (Shen et al., 2007). Anxiogenic effects mediated by α4βδ GABARs at puberty are also seen after stress-related increases in endogenous 3α,5α-THP (Shen et al., 2007).

We tested the possibility that α4βδ GABARs would be increased compensatorily in CA1 hippocampus by the stimulant actions of METH (Yamamoto et al., 1999). The unique characteristics of this receptor may explain