GROUP I METABOTROPIC GLUTAMATE RECEPTORS MODULATE ENDOCANNABINOID-MEDIATED STRESS-INDUCED ANALGESIA

by

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(Under the Direction of Andrea G. Hohmann)

ABSTRACT

Stress-induced analgesia (SIA) is mediated by mobilization of endocannabinoid lipids such as 2-arachidonoylglycerol (2-AG) in the midbrain periaqueductal gray (PAG). 2-AG may be synthesized on demand to induce SIA through the consecutive activation of two enzymes– phospholipase C (PLC) and diacylglycerol lipase (DGL). We examined whether activation of postsynaptic group I metabotropic glutamate receptors (mGluRs) would enhance SIA because of their known coupling to PLC. Microinjection of the group I mGluR agonist DHPG into the dorsolateral PAG (dPAG) enhanced SIA through a CB_1-dependent mechanism. Microinjection of the DGL inhibitors tetrahydrolipstatin (THL) and RHC80267 into the dPAG suppressed SIA. The DHPG-induced enhancement of SIA was blocked by THL. Off-site injections of the active compounds did not alter SIA. Our results support the hypothesis that exposure to environmental stressors stimulates synthesis of 2-AG through the PLC/DGL pathway to induce SIA. Moreover, this process may be initiated by activation of group I mGluRs.

INDEX WORDS: endocannabinoid, antinociception, metabotropic glutamate receptors

LIST OF ABBREVIATIONS: 2-AG, 2-arachidonylglycerol; DAG, diacylglycerol; DGL, diacylglycerol lipase; mGluR, metabotropic glutamate receptor; PLC, phospholipase C; SIA, stress-induced analgesia.

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DEDICATION

I would like to dedicate this thesis to my grandparents, James H. Brand and Maxine L. Brand, my aunts, Michelle A. Brand and Paula K. Brand, and my mother, Marsha G. Watson. Their love and support has made everything possible. Dedication also goes to Max M. Brand for having unshakeable faith in me.

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CHAPTER 1

INTRODUCTION

Environmental stressors transiently activate neural systems that suppress pain (Lewis et al., 1980) by activating descending pain inhibitory systems (Watkins et al., 1984). This phenomenon known as stress-induced analgesia (SIA) is largely mediated by endogenous opioid peptides (Akil et al., 1986). However, the antinociceptive effects of certain durations and parameters of stress cannot be blocked with opiate antagonists such as naloxone (Lewis et al., 1980). It has been shown that intermittent foot-shock induces an opioid form of stress antinociception whereas brief continuous foot-shock induces antinociception that is insensitive to blockade by opiate antagonists (Hohmann et al., 2005; Terman et al., 1983). These observations demonstrate the existence of non-opioid mechanisms of stress antinociception.

Previous work in our laboratory has recently reported that an opioid independent form of stress antinociception may be mediated by mobilization of endogenous cannabinoid lipids in the dorsal midbrain (Hohmann et al., 2005). Anandamide and 2-arachidonoylglycerol (2-AG) are the best characterized endocannabinoids identified so far. Endocannabinoids produce centrally-mediated pharmacological effects through binding to cannabinoid CB_1 receptors (for review see (Piomelli, 2003)). CB_1 receptors are heterogeneously distributed in many brain regions including the periaqueductal gray (PAG) (Herkenham et al., 1991). CB_1 receptors in the PAG regulate exogenous (Maione et al., 2006) and endogenous (Hohmann et al., 2005; Suplita et al., 2005) cannabinoid antinociception. Non-opioid stress antinociception correlates strongly with accumulation of 2-AG in the midbrain (PAG) (Hohmann and Suplita, 2006). Selective CB_1

antagonists block this antinociceptive effect consistent with mediation by CB_1. Moreover, inhibition of 2-AG deactivation enhances both 2-AG accumulation and stress antinociception through a cannabinoid CB_1-dependent mechanism (Hohmann et al., 2005).

Endocannabinoids are believed to act as retrograde messengers (Wilson and Nicoll, 2001). Consistent with this hypothesis, CB_1 receptors are localized to presynaptic sites on GABAergic and glutamatergic neurons (Katona et al., 1999; Katona et al., 2006). Endocannabinoid binding to CB_1 receptors would therefore be expected to inhibit the release of the primary neurotransmitter. Thus, it is noteworthy that activation of CB_1 receptors by exogenous cannabinoids inhibits GABAergic and glutamatergic synaptic transmission in the rat PAG (Vaughan et al., 2000).

In vitro studies suggest that 2-AG biosynthesis occurs through the consecutive activation of phospholipase C (PLC) and diacylglycerol lipase (DGL) as depicted in Figure 1 (Bisogno et al., 1997; Jung et al., 2007). *In vitro* studies also suggest that activation of group I metabotropic glutamate receptors (mGluRs), which are positively coupled to PLC (Pin and Duvoisin, 1995), depresses synaptic transmission through a CB_1 –dependent mechanism. These studies support the hypothesis that activation of mGluRs mobilizes an unidentified cannabinoid mediator that may be 2-AG (Chevaleyre and Castillo, 2003; Gerdeman and Lovinger, 2001; Riegel and Lupica, 2004). Activation of group I mGluRs increases 2-AG levels in brain hippocampal cells (Jung et al., 2005) *in vitro*, suggesting that activation of these receptors initiates the biosynthesis of 2-AG. *In vivo* studies demonstrate that activation of group I mGluRs in the PAG produces antinociception (Maione et al., 2000).

2-AG biosynthesis and mobilization also requires the enzyme DGL (Hashimotodani et al., 2007; Katona et al., 2006). Two isoforms of DGL (DGL- α and DGL-β) have been identified.

DGL-α, the more abundant of the two DGL isoforms expressed in the adult mouse brain, is absent in axonal tracts and localizes to postsynaptic dendrites in cerebellar Purkinje cells (Bisogno et al., 2003). DGL-α is also concentrated in a perisynaptic compartment of the dendritic spine which is known to contain group I metabotropic glutamate receptors (Lujan et al., 1996). Mobilization of 2-AG produces pharmacological effects that are dependent upon the CB_1 receptor (Bisogno et al., 2003; Palomaki et al., 2007) or CB_2 receptor (Guindon et al., 2007). *In vitro* studies also suggest that DGL-mediated hydrolysis of diacylglycerol (DAG) is implicated in 2-AG biosynthesis (Bisogno et al., 2003; Hashimotodani et al., 2007). However whether these same pathways control 2-AG biosynthesis *in vivo* remains unknown.

The present study was conducted to investigate the mechanisms controlling 2-AG mobilization under physiological condition using a model of endocannabinoid-mediated stress antinociception. We hypothesized that stress stimuli that result in non-opioid stress antinociception stimulate formation of 2-AG through the PLC/DGL pathway to induce antinociception. We tested the hypothesis that stimulation of mGluRs, which are positively coupled to PLC (Pin and Duvoisin, 1995), would enhance stress antinociception. We also tested the hypothesis that inhibition of DGL would suppress stress antinociception. Finally, we examined whether the enhancement of stress antinociception produced by activation of group I mGluRs could be blocked through inhibition of DGL. Elucidating the mechanism governing 2-AG mobilization in the brain under physiological conditions may facilitate the development of novel endocannabinoid-based therapeutic interventions.

CHAPTER 2

MATERIALS AND METHODS

Subjects

One hundred and forty eight adult male Sprague-Dawley rats were used in these experiments. All procedures were approved by the University of Georgia Animal Care and Use Committee and followed the guidelines for the treatment of animals of the International Association for the Study of Pain (Zimmermann, 1983).

Surgical Procedures

Prior to surgery, rats were anesthetized through i.p. administration with a sodium pentobarbital and ketamine mixture (25 mg/kg and 40 mg/kg, respectively). The rat brain atlas of (Paxinos and Watson, 1998) was used to obtain stereotaxic coordinates for cannulae implantation. Stainless steel guide cannulae (24 g; Small Parts, Inc. Miami, FL) were implanted above the dPAG (-5.35 mm DV, +1.6 mm AP, +0.67 mm LM) or deliberately off-site using the skull surface landmark lambda as the zero point. Stainless steel insect pins were used to prevent occlusion of the cannulae before and after microinjections. Animals were allowed to recover five to seven days prior to testing.

Drug preparation and administration

DHPG was obtained from Tocris Cookson (Ellisville, MO). Rimonabant (SR141716A) was a gift from NIDA. Tetrahydrolipstatin (THL) was purchased from Sigma Aldrich (St. Louis, MO). RHC80267 was ordered from Calbiochem (San Diego, CA). Drugs were dissolved in

DMSO and delivered intracranially (1 μl volume). Intracranial injections were performed using a microinjection pump at a speed of 30nl/s.

Behavioral testing

Stress-induced analgesia was induced by exposing rats to a continuous foot-shock stressor for 3 min (0.9 mA, AC current) as described previously (Connell et al., 2006; Hohmann et al., 2005; Suplita et al., 2005; Suplita et al., 2006; Suplita et al., 2007). Stress antinociception was quantified behaviorally using the tail-flick test (D'Amour, 1941). The latency for rats to remove their tails from a radiant heat source was measured (IITC Inc., Model 33A, Woodland Hills, CA). Rats were habituated to restraining tubes for 15 minutes prior to testing. A 10-second cut-off latency was used to prevent tissue damage. Stable baseline withdrawal responses to the heat source were established before the animals received an intracranial injection of drug or vehicle. Immediately following microinjection, tail-flick latencies were assessed three times at 2-minute intervals and averaged for each rat to assess any changes in nociceptive responding induced by the injection alone. Rats were subsequently exposed to brief continuous foot-shock for 3 minutes at 0.9mA. Tail-flick latencies were measured after foot-shock in 2-minute intervals for 60 minutes. Intracranial injections were performed 5 minutes prior to foot-shock. The experimenter was blinded to the experimental condition in all studies.

Experiment 1: To determine whether activation of group I metabotropic glutamate receptors enhanced stress antinociception, DHPG (0.02 μg or 0.2 μg) or vehicle was microinjected into the dPAG. To assess pharmacological specificity, separate groups received DHPG (0.2 μg) co-administered with rimonabant (0.1 μg), administered at a dose which did not alter stress antinociception (Hohmann et al., 2005; Suplita et al., 2006). Tail-flick latencies were measured before and after exposure to 3 min of continuous foot-shock as described above.

Experiment 2: To determine whether inhibition of DGL suppressed stress antinociception, THL (0.1, 1 or 10 μg), RHC80267 (1, 10 or 100 μg) or vehicle was microinjected into the dPAG. Tail-flick latencies were measured before and after exposure to 3 min of continuous foot-shock as described above.

Experiment 3: To determine whether activation of group I metabotropic glutamate receptors enhance stress antinociception through activation of the PLC/DGL pathway, separate groups received DHPG (0.02 or 0.2 μg) or DHPG co-administered with THL (0.1 μg). In this study DHPG was administered at the dose shown in Experiment 1 to enhance stress antinociception, whereas THL was administered at a dose that failed to attenuate stress antinociception in Experiment 2. Tail-flick latencies were measured before and after exposure to 3 minutes of continuous foot-shock as described above.

Histology

Rats were deeply anesthetized with sodium pentobarbital (65 mg/kg i.p.) and then perfused transcardially with 0.9% saline followed by 10% formalin. The brains were dissected out and fixed for at least 24 hours in 30% sucrose. Coronal sections (40 μm) through the PAG were cut using a cryostat and mounted onto gelatin-subbed slides. Slides were stained with cresyl violet and cover-slipped. Injection sites were confirmed under a light microscope by an investigator blinded to the experimental condition.

Statistical analysis

Behavioral data were analyzed by repeated measures Analysis of Variance (ANOVA), ANOVA and analysis of covariance (ANCOVA) as appropriate. Post hoc comparisons were evaluated using Fisher's Protected Least-Squares Difference (LSD) to correct for inflated alpha error, with $P < 0.05$ considered as statistically significant.

CHAPTER 3

RESULTS

General experimental results

In all studies, baseline tail-flick latencies, measured prior to drug or vehicle administration, did not differ in any of the groups prior to exposure to foot-shock. Post-injection tail-flick latencies, assessed after microinjections but prior to foot-shock were also similar between groups, with one exception (described in Experiment 2 below).

Experiment 1: Activation of group I metabotropic glutamate receptors enhances stress antinociception through a CB_1-dependent mechanism

Microinjection of DHPG into the dPAG (Fig. 2B) enhanced the antinociceptive effect produced by exposure to foot-shock ($F_{2,15} = 7.137$, $P < 0.008$; Fig.2A) and differentially altered the time course of stress antinociception ($F_{30,210} = 2.308$, $P < 0.0004$; Fig. 2A). Post hoc comparisons revealed that stress antinociception was greater in groups receiving either the low ($p < 0.007$) or the high dose of DHPG ($p < 0.006$) relative to groups receiving the vehicle. By contrast, off-site microinjection of the high dose of DHPG (Fig. 3B) did not alter stress antinociception (Fig. 3A).

The DHPG-induced enhancement of foot shock-induced antinociception was blocked by the CB_1 receptor antagonist rimonabant ($F_{3,20} = 4.588$, $P < 0.02$; Fig. 4A). Stress antinociception was greater in animals receiving DHPG compared to those receiving rimonabant ($P < 0.02$), vehicle ($P < 0.006$), or rimonabant co-administered with DHPG ($P < 0.005$). The rimonabant-

induced blockade of the DHPG-induced enhancement of stress antinociception was also time dependent ($F_{45,300} = 2.527$, $P < 0.0002$). Microinjection sites are shown in Fig. 4B.

Experiment 2: Microinjection of DGL inhibitors in the dPAG suppresses stress antinociception

Microinjection of the DGL inhibitor RHC80267 into the dPAG (Fig.5B) induced a time-dependent ($F_{27,198} = 3.308$, $P < 0.0002$; Fig.5A) suppression of stress antinociception. The DGL inhibitor THL also produced a dose-dependent suppression of stress antinociception relative to vehicle controls ($F_{3,26} = 5.086$, $P < 0.007$; Fig.6A). The high dose of THL suppressed stress antinociception relative to either DMSO ($p < 0.005$) or the lowest dose (0.1 μg) of THL ($p < 0.003$). This suppression was time-dependent ($F_{27,234} = 5.598$, $P < 0.0001$). The low dose of THL (0.1 μg) was not sufficient to suppress stress antinociception. The high and middle doses of THL (10 μg and 1 μg, respectively) suppressed stress antinociception with equivalent magnitudes. However, the suppression of stress antinociception induced by the high dose of THL outlasted that of the middle dose of THL (Fig. 6A). Microinjection sites are shown in Fig.6B.

Prior to foot-shock, off-site microinjection of THL and RHC produced a modest but significant increase in tail-flick latencies with respect to the vehicle control group ($F_{2,20} = 5.016$, $P < 0.04$; $P < 0.05$ for each comparison). ANCOVA was therefore used to assess group differences in stress antinociception to remove the effects of microinjection-induced alteration of post-injection tail-flick latencies on stress antinociception. Post-injection tail-flick latencies, determined prior to exposure to foot-shock, were used as the covariate. ANCOVA failed to reveal changes in stress antinociception following off-site injection of THL or RHC relative to vehicle. Off-site microinjections of the DGL inhibitors RHC and THL also failed to alter stress antinociception compared to vehicle controls (Fig. 7A, B).

Experiment 3: Activation of group I metabotropic glutamate receptors enhances stress antinociception through the PLC/DGL pathway

The enhancement of stress antinociception induced by DHPG (0.02 μg and 0.2 μg) was blocked in a time-dependent manner by THL, administered at a dose that was insufficient to suppress stress antinociception ($F_{15,150} = 5.243, P < 0.0002$ and $F_{15,210} = 6.277, P < 0.0002$, in Fig. 8A and 8C, respectively). Stress antinociception was greater in groups receiving the high dose of DHPG (0.2 μg) relative to groups receiving DHPG co-administered with THL across the entire observation interval ($F_{1,14} = 16.065$, $P < 0.002$). Post hoc analysis confirmed that stress antinociception was lower in groups receiving THL co-administered with DHPG relative to groups receiving DHPG alone ($p < 0.002$). Microinjection sites are shown in Fig.8B and 8D.

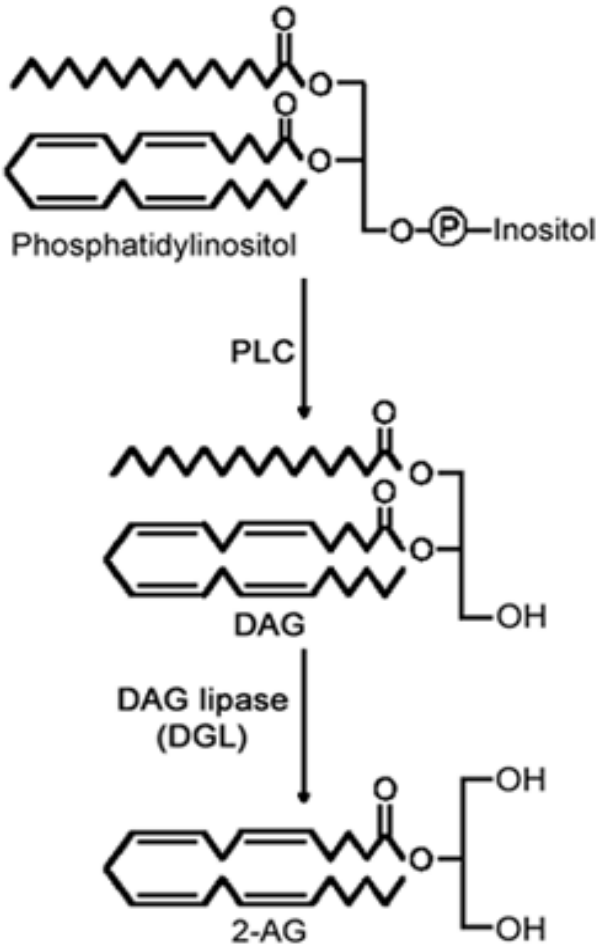
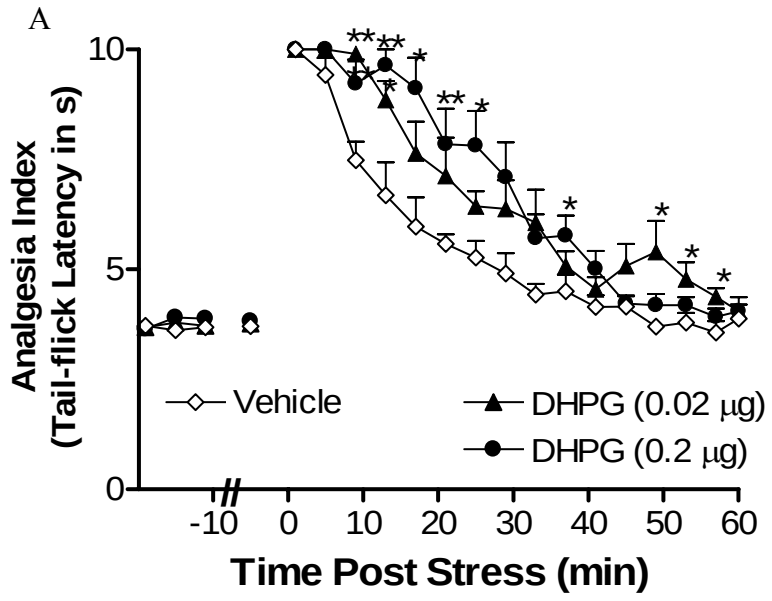


Figure 1. The hypothetical pathway of 2-AG formation. 2-AG may be synthesized through successive activation of two enzymes- PLC and DGL. DAG is formed from PLC-medieated hydrolysis of membrane phospholipids precursors. DAG is subsequently hydrolyzed by DGL to generate 2-AG.



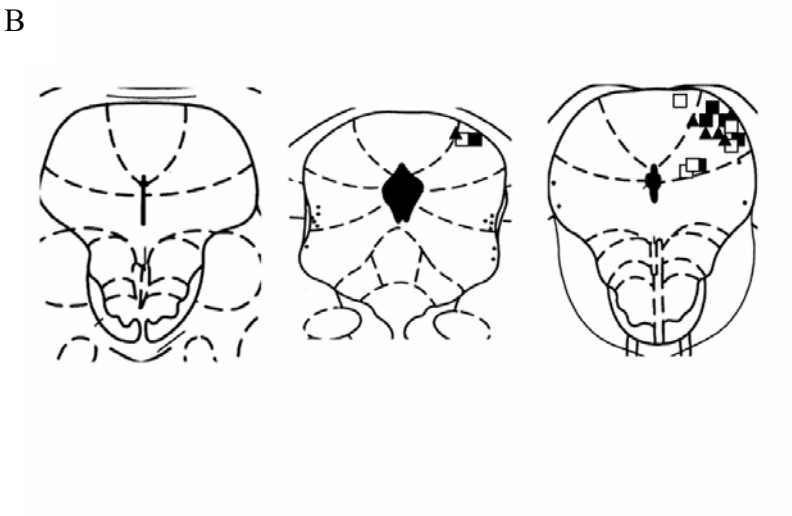
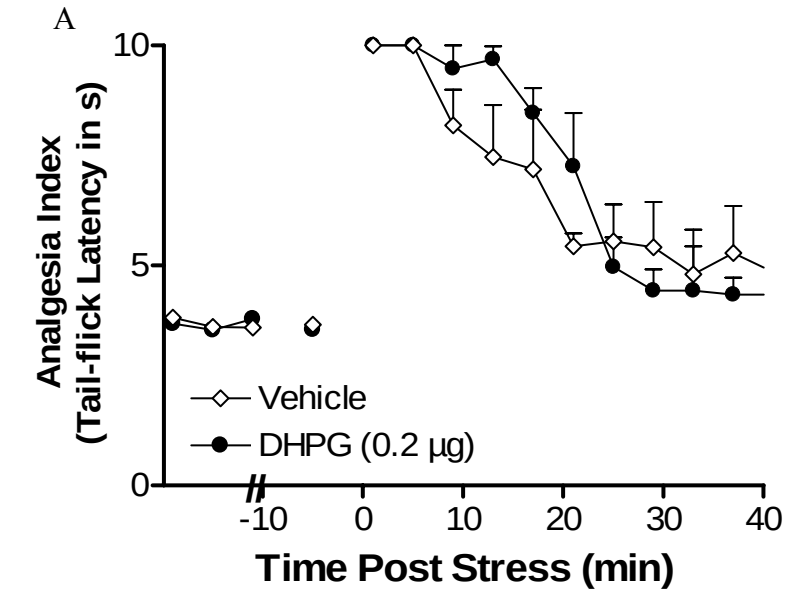


Figure 2. (A) Microinjection of the group I mGluR agonist DHPG into the dPAG enhances SIA. (B) Microinjection sites for data shown in Figure 2A for groups receiving 0.02 μg DHPG (■), 0.2 μg DHPG (▲) and DMSO (□).



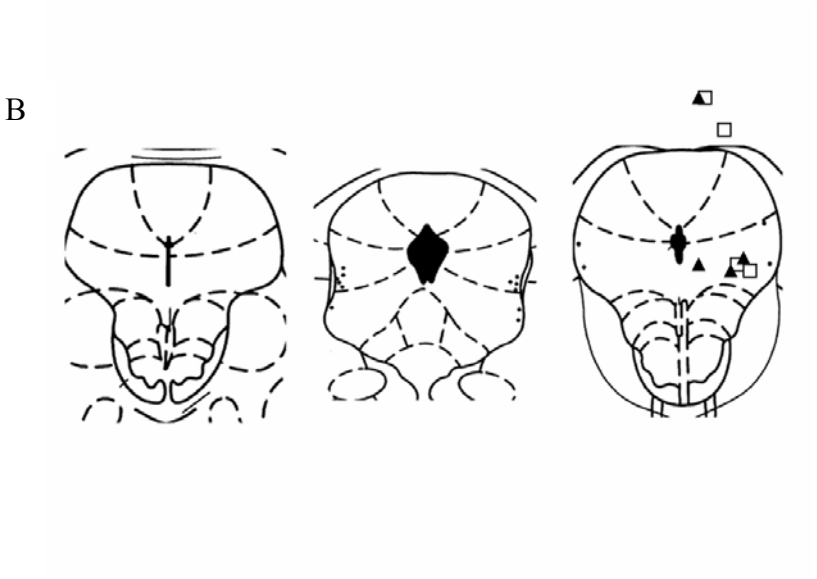


Figure 3. (A) Microinjection of the group I mGluR agonist DHPG off-site of the dPAG does not alter SIA. (B) Microinjection sites for data shown in Fig. 3A for groups receiving 0.2 µg DHPG (▲) and DMSO (□).

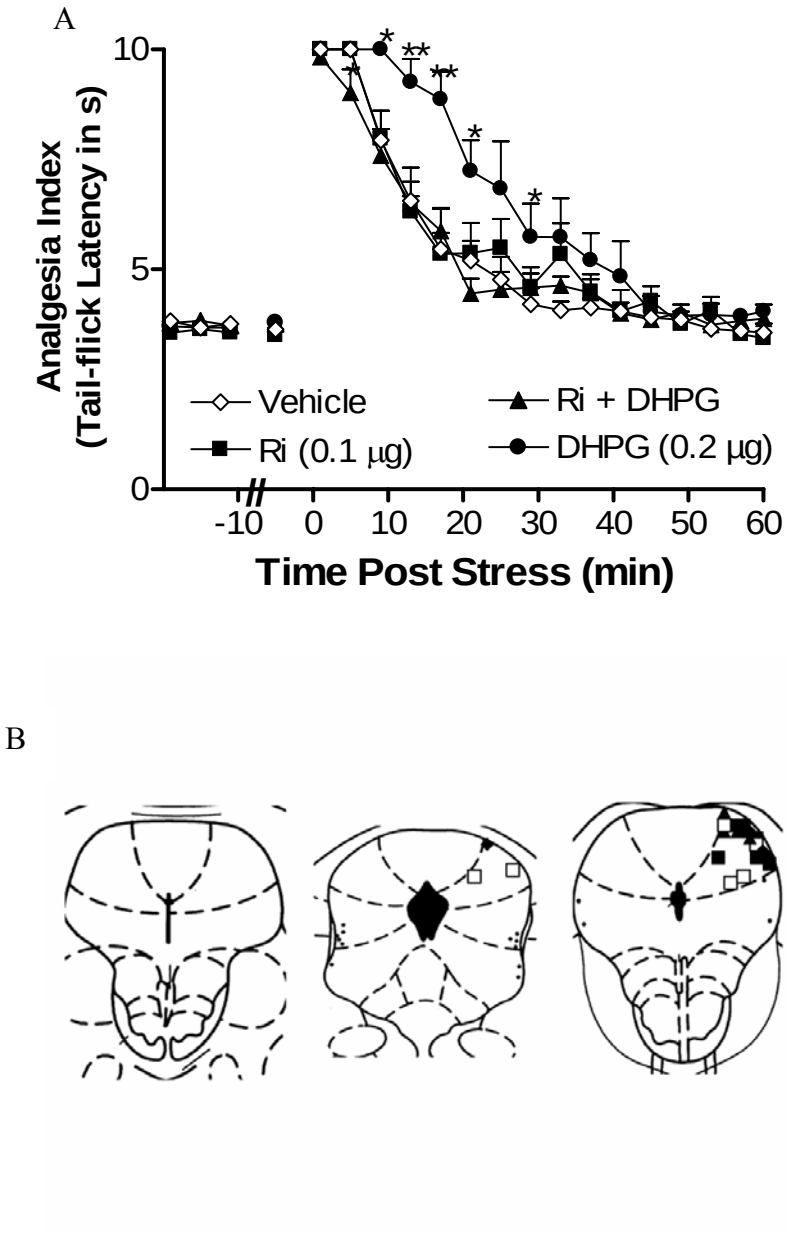


Figure 4. (A) The enhancement of SIA induced by microinjection of DHPG (0.2 μg) into the dPAG is blocked by the CB_1 antagonist rimonabant (Ri). Rimonabant was co-administered with DHPG. (B) Microinjection sites in the dPAG for groups receiving 0.2 μg DHPG (■), 0.1 μg Ri (♦), DHPG + Ri (▲), DMSO (□).

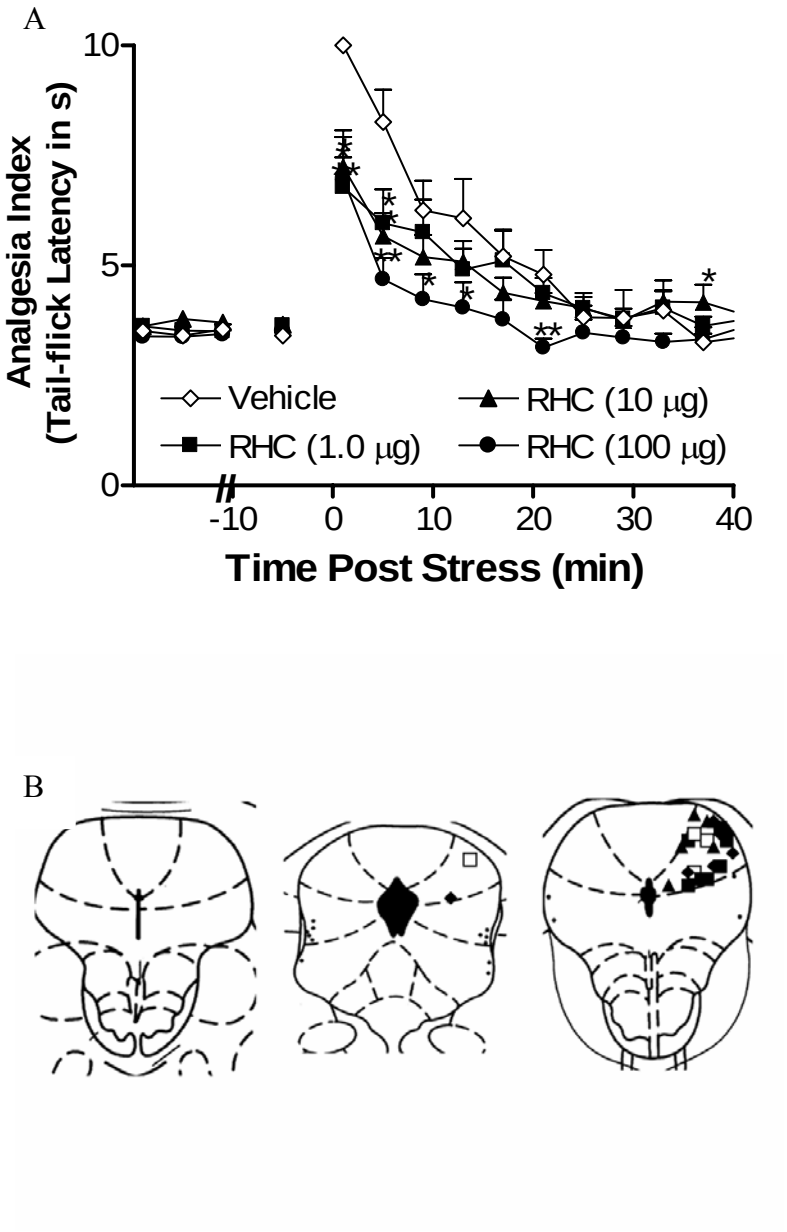
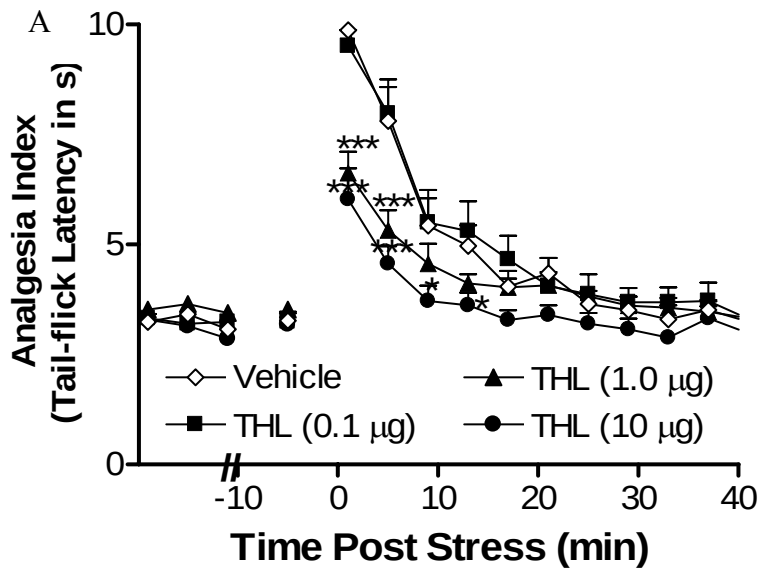


Figure 5. (A) The DGL inhibitor RHC80267 (RHC) suppresses SIA following microinjection into the dPAG. (B) Microinjection sites of in the dPAG for groups receiving 1 μg RHC (■), 10 μg RHC (▲), 100 μg RHC (♦), DMSO (□).



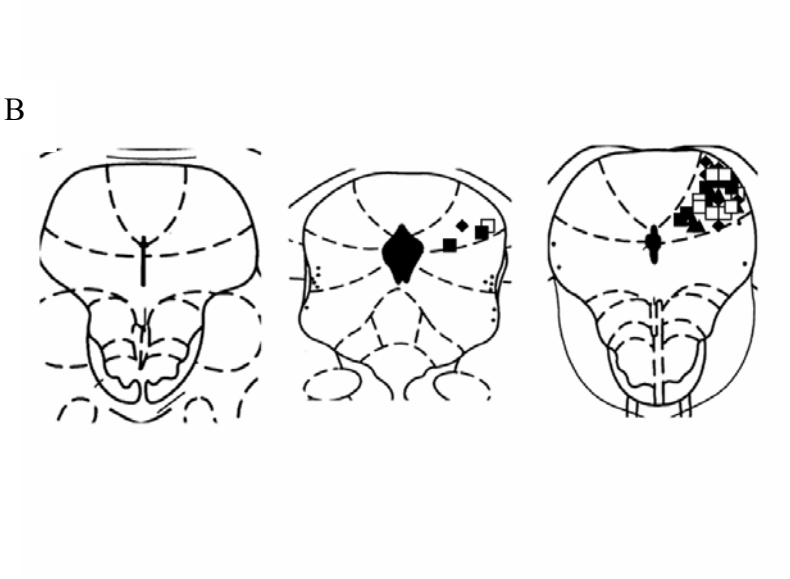


Figure 6. (A) The DGL inhibitor tetrahydrolipstatin (THL) suppresses SIA following microinjection into the dPAG. (B) Microinjection sites in the dPAG for groups receiving 0.1 μg THL (♦), THL 1 μg (▲), THL 10 μg (■) and DMSO (□).

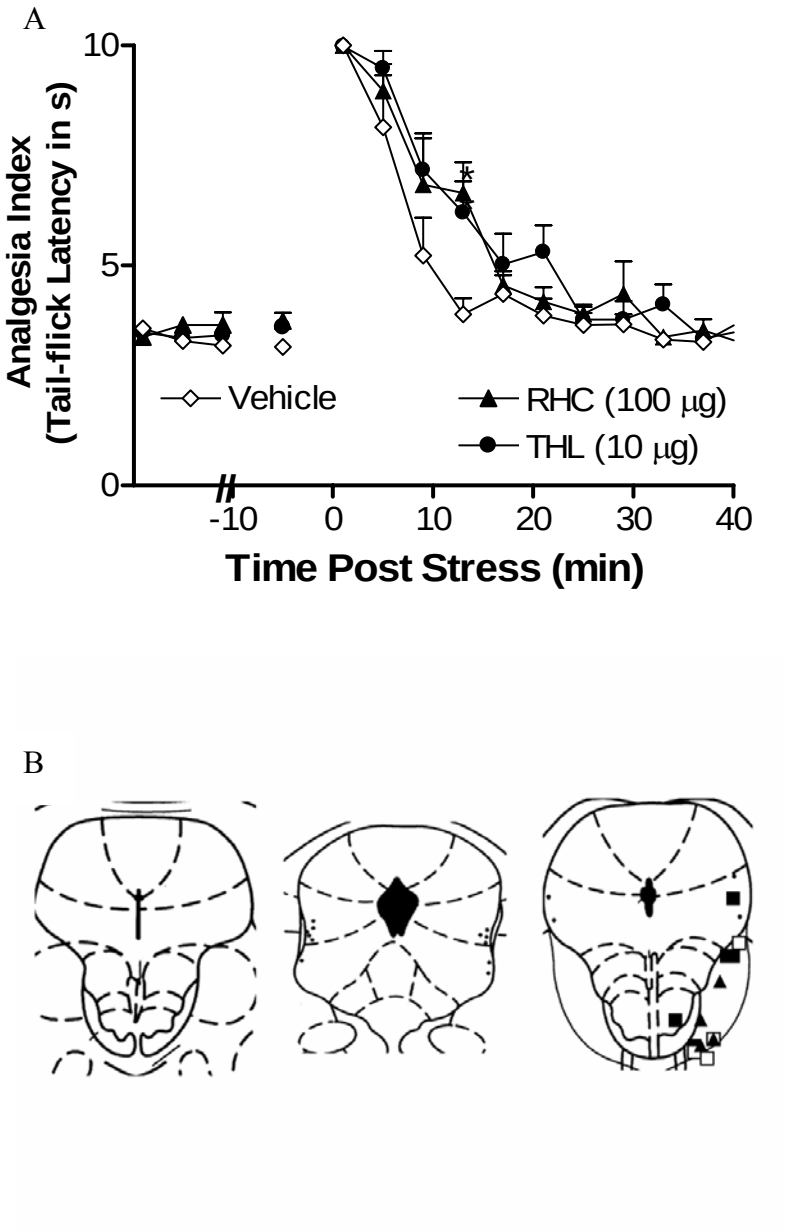


Figure 7. (A) Off-site microinjections of RHC and THL do not alter stress antinociception. (B) Sites of microinjections of 100 μg RHC (♦), 10 μg THL (■) and DMSO (□).

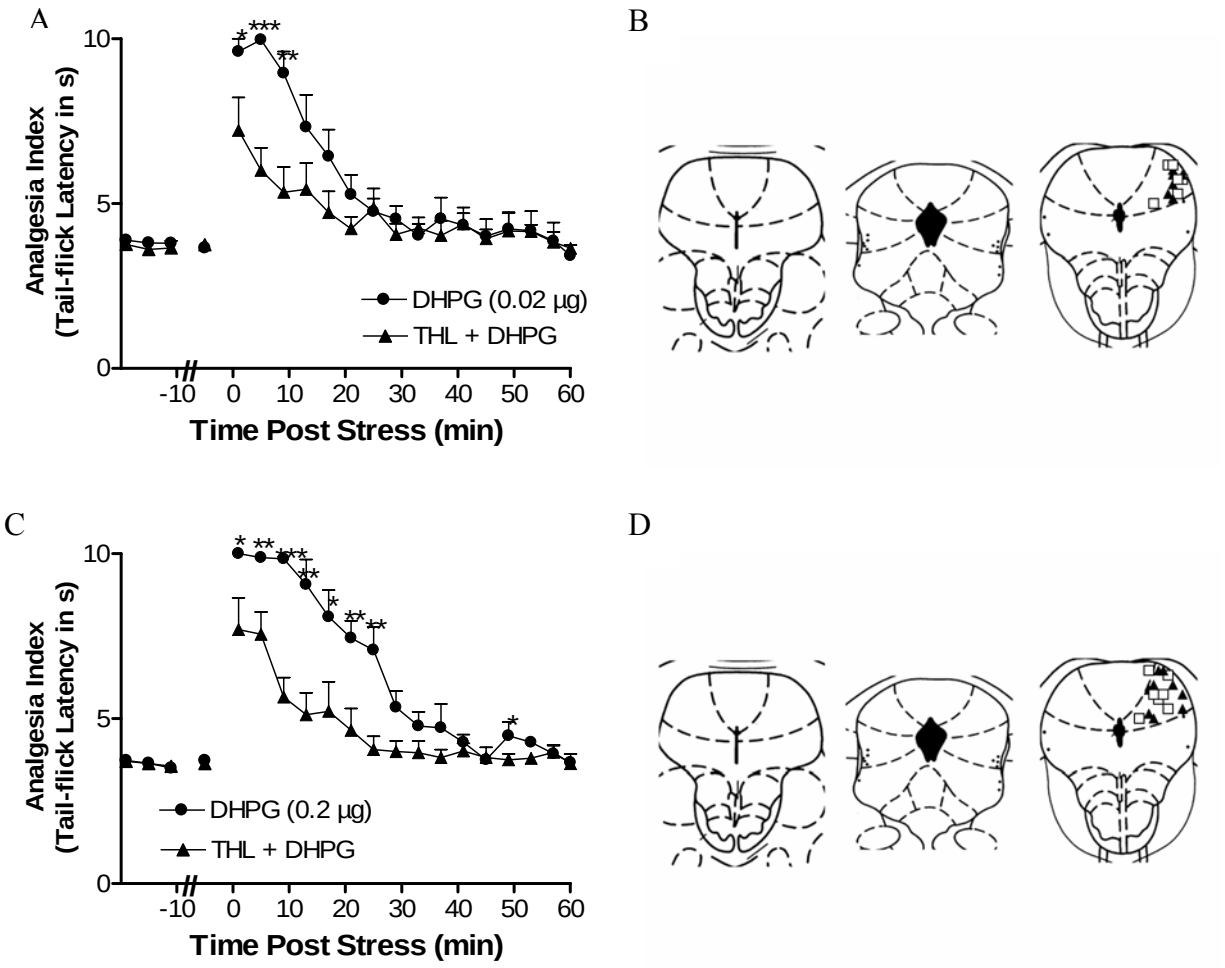


Figure 8. The DGL inhibitor THL blocks the enhancement of SIA induced by DHPG (0.02 µg) (A) and DHPG (0.2 µg) (C). Microinjection sites in the dPAG for groups receiving (B) 0.02 µg DHPG (▲) and DHPG + THL (□) or (D) 0.2 µg DHPG (▲) and DHPG + THL (□).

CHAPTER 4

DISCUSSION

Exogenous cannabinoids produce antinociception following either systemic (Lichtman and Martin, 1991) or local injection into brain regions implicated in the descending control of pain. For example, microinjection of synthetic cannabinoids into either the PAG (Finn et al., 2003; Lichtman et al., 1996; Martin et al., 1995) or rostralventromedial medulla (Martin et al., 1998; Meng et al., 1998) produces antinociception in multiple assays. The antinociceptive and electrophysiological effects of systemically administered cannabinoids are attenuated following spinal transection (Hohmann et al., 1999; Lichtman and Martin, 1991), demonstrating the importance of supraspinal sites of action to cannabinoids antinociceptive mechanisms. The present data suggest that the dPAG is also a site of action implicated in both endocannabinoid-mediated stress-induced analgesia as well as 2-AG biosynthesis. In our study, injections of group I mGluR agonists and multiple inhibitors of DGL into the dorsolateral PAG modulated stress antinociception, but off-site injections were ineffective. These studies further emphasize the importance of the PAG in endogenous as well as exogenous antinociceptive mechanisms.

Activation of CB_1 receptors, which in the PAG induces antinociception, inhibits GABAergic and glutamatergic synaptic transmission, thereby controlling the descending pain pathway (Maione et al., 2006; Vaughan et al., 2000). Previous work in our lab implicates a role for endocannabinoids in the dorsolateral PAG in stress antinociception (Hohmann et al., 2005; Suplita et al., 2005). A strong correlation is observed between 2-AG levels in the dorsal midbrain fragments that contain the intact periaqueductal gray and endocannabinoid-mediated stress

antinociception (Hohmann et al., 2005). Moreover, inhibition of 2-AG deactivation increases levels of 2-AG in the same brain region and induces a CB_1-mediated enhancement of stress antinociception (Hohmann et al., 2005).

The pharmacological approach employed in the present study suggests that activation of group I mGluRs in the PAG I may trigger the mobilization of 2-AG to induce stress antinociception. *In vitro* studies have shown that 2-AG biosynthesis occurs through the PLC/DGL pathway (Bisogno et al., 1997; Jung et al., 2005). Activation of postsynaptic group I mGluRs, which are positively coupled to PLC (Pin and Duvoisin, 1995), induce cannabinoid receptor-mediated changes in GABAergic synaptic efficacy in hippocampal cells (Chevaleyre and Castillo, 2003). These studies suggest that activation of mGluRs induces the mobilization of an unidentified endocannabinoid mediator to modulate neuronal excitability.

In our study, activation of group I mGluRs in the dPAG enhanced the antinociceptive effects produced by exposure to an environmental stressor. Activation of group I mGluRs with DHPG, administered intracranially in the dPAG, enhanced stress antinociception in the tail-flick test. This concurs with previous findings which show that activation of group I metabotropic glutamate receptors in the PAG causes antinociception (Jung et al., 2005; Maione et al., 2000). These findings are also consistent with previous *in vitro* work suggesting that activation of group I mGluRs promotes the biosynthesis of 2-AG (Jung et al., 2005).

In our study, the CB_1 receptor antagonist rimonabant blocked the enhancement of stress antinociception produced by the group I metabotropic glutamate receptor agonist DHPG. These data support our hypothesis that activation of mGluRs enhances stress antinociception through a mechanism that requires CB_1 receptor activation. Importantly, microinjection of rimonabant blocked the enhancement of stress antinociception induced by activation of group I mGluRs at a

dose that was not sufficient to suppress stress antinociception. This observation coincides with *in vitro* studies showing a functional link between postsynaptic group I mGluRs and presynaptic CB_1 receptors (Maejima et al., 2001).

Two subtypes of group I mGluRs have been described, mGluR1 and mGluR5 (Pin and Duvoisin, 1995). Recent studies (Jung et al., 2005; Palazzo et al., 2001), together with unpublished data from our lab, specifically implicate a role for group I mGluR5 in triggering 2-AG biosynthesis. A group I mGluR5 antagonist but not a group I mGluR 1 antagonist suppressed endocannabinoid-mediated stress antinociception in the present model (Bolton and Hohmann, unpublished). More work is necessary to demonstrate that the enhancement of stress antinociception induced by the group I mGluR agonist DHPG is blocked by a group I mGluR5 antagonist.

Diacylglycerol lipase is a major enzyme regulating 2-AG biosynthesis and mobilization (Hashimotodani et al., 2007; Katona et al., 2006). 2-AG thus mobilized produces pharmacological effects that are dependent upon the CB_1 receptor (Bisogno et al., 2003; Palomaki et al., 2007). In our study, pharmacological inhibition of DGL with both RHC and THL, through intracranial microinjection into the dPAG, suppressed stress antinociception immediately following termination of the stressor. The fact that similar suppressions of stress antinociception were observed with structurally distinct DGL inhibitors suggests that local administration of DGL inhibitors suppressed stress antinociception by decreasing 2-AG synthesis. These findings are also consistent with previous *in vitro* studies demonstrating that 2-AG is formed through the phospholipase C/diacylglycerol lipase pathway (Bisogno et al., 2003; Bisogno et al., 1997; Hashimotodani et al., 2007; Jung et al., 2005).

Off-target effects of DGL inhibitors have recently been reported (Ghisdal et al., 2005; Palomaki et al., 2007). However, three complementary observations suggest that the effects of RHC and THL in our model were mediated by inhibition of DGL. First, structurally distinct DGL inhibitors induced similar suppressions of stress antinociception, suggesting a common mechanism of action. Second, the time-course of suppression of stress antinociception following microinjection into the dPAG suggests that the DGL inhibitors suppressed stress antinociception specifically during the interval when 2-AG is mobilized (Hohmann and Suplita, 2006; Hohmann et al., 2005). Both DGL inhibitors specifically inhibited endocannabinoid-mediated stress antinociception during the early but not the late phase of stress antinociception. At this time, 2-AG is mobilized by exposure to the foot-shock stressor but anandamide levels remain unchanged. Third, the effects of the group I mGluR agonist DHPG were blocked by THL, an effect which is consistent with the actions of THL in inhibiting DGL, but not with more recently described off-target effects of THL (Palomaki et al., 2007). The suppression of stress antinociception induced by intra-PAG injection of RHC and THL is consistent with the hypothesis that local administration of the DGL inhibitors suppressed stress antinociception by decreasing 2-AG biosynthesis. These findings are also consistent with previous *in vitro* studies implicating that 2-AG is formed through the PLC/DGL pathway (Jung et al., 2005) and that DGL hydrolysis of DAG may be the first committed step of 2-AG biosynthesis (Bisogno et al., 2003).

In conclusion, activation of group I mGluRs with DHPG in the dPAG enhances stress antinociception through a CB_1 -dependent mechanism. We have also found that inhibition of the enzyme DGL with two distinct inhibitors, RHC80267 and THL, suppresses endocannabinoid-mediated stress antinociception. THL also suppresses the enhancement of stress antinociception

induced by activation of group I mGluRs with DHPG. These findings lead us to conclude that endocannabinoid-mediated stress antinociception may be initiated by activation of postsynaptic group I mGluRs. In this model, activation of group I mGluRs initiates a cascade of events by activating the PLC/DGL pathway in the dPAG, presumably by triggering the biosynthesis of 2-AG. Further studies, which employ high performance liquid chromatography/mass spectrometry to measure levels of endocannabinoids following microinjection of behaviorally active doses of DHPG and THL into the dPAG, are required to definitively test this hypothesis.

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