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13. ABSTRACT (Maximum 200 Words)

The experiments under Specific Aim 1 were continued and the work related to Hypothesis 1 under this aim was completed. In these experiments dual-probe microdialysis was performed with one probe in the ventral tegmental area (VTA) and the other in the ipsilateral nucleus accumbens (NACC). CP 93129 (20, 40, and 80 μM), a 5-HT1B receptor agonist, was infused into the VTA of separate groups of rats. Dialysates from the VTA and NACC were collected for assay of dopamine (DA) and GABA. In another experiment, SB-216641 (10 μM, a 5-HT1B receptor antagonist), BRL-15572 (10 μM, a 5-HT1D/1A receptor antagonist), or WAY-100635 (10 μM, a 5-HT1A receptor antagonist) was infused into the VTA for 40 min, and then co-infused with CP 93129 (80 μM) for another 60 min. The results showed that intra tegmental CP 93129 increased DA concentrations in the VTA in a concentration-dependent manner. Administration of CP 93129 at 80 μM, but not 20 or 40 μM, into the VTA also significantly decreased GABA in this region. Co-infusion of SB-216641, but not BRL-15572 or WAY-100635, antagonized not only the effects of intra-tegmental CP 93129 (80 μM) on VTA DA and NACC DA but also on VTA GABA. The results suggest that activation of VTA 5-HT1B receptors increases mesolimbic DA neuron activities. The increased DA neuronal activity may be associated, at least in part, with the 5-HT1B receptor-mediated inhibition of VTA GABA release. The work related to Specific Aim 2 was also started in Year 2. After several months of efforts, the method of genotyping 5-HT1B receptor knockout (KO) and their counterparts wild-type (WT) mice has been developed and a number of KO and WT mice were generated at the PI's lab. Microdialysis experiments have already been started in these mice.

14. SUBJECT TERMS

Ethanol, Dopamine, Serotonin, 5HT-1B receptor, GABA, nucleus accumbens, Ventral tegmental area, Knockout mice

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INTRODUCTION

The purpose of this project entitled "Ethanol and mesolimbic serotonin (5-HT)/dopamine (DA) interactions via 5-HT-1B receptors" is to investigate whether activation of 5-HT1B receptors within the ventral tegmental area (VTA) facilitates DA transmission in the ipsilateral nucleus accumbens (NACC) and potentiates ethanol-induced increases in NACC DA by 5-HT1B receptor-mediated GABA mechanisms. The scope of this project covers the following specific aims: (1) To determine the involvement of 5-HT_{1B} heteroreceptors on GABA terminals in the VTA in the modulation of GABA release in the VTA and DA release in the ipsilateral NACC, and its involvement in the neurochemical effect of acute ethanol in freely moving animals; (2) To compare the impact of 5-HT_{1B} receptor activation on DA transmission in the NACC and on ethanol's neurochemical effects between 5-HT_{1B} receptor knock-out (KO) mice and their counterparts wild-type (WT) mice, (3) To determine the involvement of 5-HT_{1B} heteroreceptors on GABA terminals in the VTA in the modulation of DA and GABA releases in the VTA, and its involvement in the effect of ethanol in superfused VTA slices.

BODY

According to Statement of Work, experiments for Specific Aim 2 will be started in Year 3 and performed in 5-HT1B receptor knock-out (KO) and their counterparts wild-type (WT) mice. However, due to unforeseen changes in the KO mouse provider, an adjustment of the progress schedule was made during the second year of the project. That is, the experiments related to Specific Aim 2 was started in Year 2, earlier than the originally proposed schedule (Year 3) in Statement of Work. Therefore, the experiments related to both Specific Aims 1 and 2 were conducted simultaneously during the period of Year 2. These changes in the sequence of the work have no negative impacts on the whole project because there is no direct sequential relationship between Specific Aim 1 and Aim 2.

Specific Aim 2: To compare the impact of 5-HT_{1B} receptor activation on DA transmission in the NACC and on ethanol's neurochemical effects between 5-HT_{1B} receptor knock-out (KO) mice and their counterparts wild-type (WT) mice

When preparing the grant application, Dr. Rene Hen at Columbia University promised to be responsible for providing approximately 120 KO and 120 WT mice for the use in the project. However, a recent spread of mouse hepatitis virus at his laboratory animal care facility has made it impossible for him to provide the animals in the near future. To solve this unforeseen problem, after I was informed of this situation by Dr. Hen in March of 2003, I contacted Dr. Irwin Lucki, a professor of University of Pennsylvania who has a separate 5-HT-1B KO mouse colony in his lab. Dr. Lucki kindly agreed to provide two KO breeders to me and the breeders were shipped to my lab at the end of August of 2003. Therefore, the work related to breeding of the KO colony was necessarily started immediately after arrival of the breeders.

Based on Dr. Hen's suggestions, in order to insure that knockout strains do not genetically drift away from their wild-type controls it is important to interbreed heterozygotes rather than homozygotes to generate KO and WT mice[1]. This practice requires genotyping all offspring because only approximately ¼ of offspring would be KO or WT. However, the technique of genotyping was new to us, as a result, somewhat difficult for us to perform because neither the post-doc nor I had backgrounds of molecular biology and because my lab was not previously equipped for this method.

After several months of our great efforts, the method of genotyping has now successfully been established and 20 WT and 13 KO mice have already been produced at my lab. These mice are old enough for the use and the microdialysis experiments that had been proposed in Statement of Work have already been started in these mice. This is one of our big accomplishments during Year 2. However, breeding and genotyping, both of which were not originally proposed in the grant application, would substantially increase our workload, and consequently to some extent, have impacts on the speed of the progress of the project.

Briefly, the method of genotyping we used is described as follows. Mouse tail DNA was extracted and purified using DNeasy Tissue Kits (Qiagen). One-three μl of DNA extracts were then directly used in the polymerase chain reaction (PCR). The following primers were used in the PCR: (1) 5'-CTT CTA TCG CCT TCT TGA CG-3'; (2) 5'-CCC ATC AGC ACC ATG TAC AC-3'; (3) 5'-GAC TTG GTT CAC GTA CAC AG-3'. PCR amplification was performed as follows: a denaturation step of 2 min at 95^0C was followed by amplification for 35 cycles (94^0C for 90 sec, 55^0C for 120 sec and 72^0C for 120 sec) and by a final step at 72^0C for 10 min. The PCR products were separated by electrophoresis in 1.5% agarose gel in 1 x TBE (Tris-Borate-EDTA) buffer containing 0.005% ethidium bromide.

Expected sizes of PCR products were: WT types: 560 bp, KO types: 680 bp, and heterozygote types: 560 bp plus 680 bp. A representative result of genotyping is listed below:

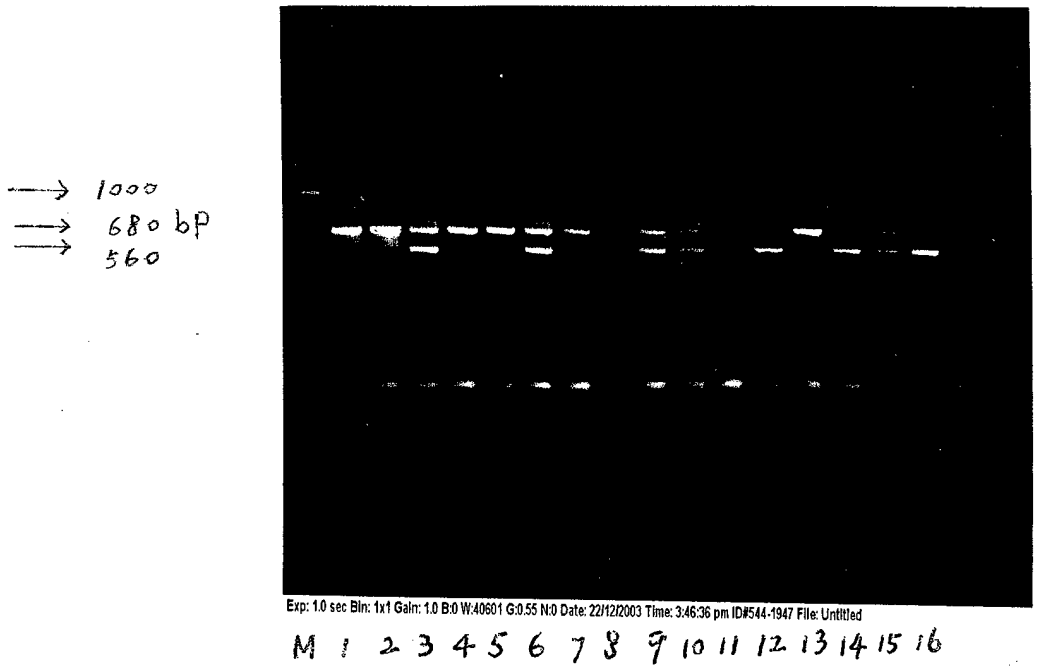

Fig 1. A representative of genotyping result. M = DNA marker, 1=KO, 2=KO, 3=Heterozygote, 4=KO, 5=KO, 6= Heterozygote, 7=KO, 8= Heterozygote, 9= Heterozygote, 10= Heterozygote, 11= Heterozygote, 12=WT, 13=KO, 14=WT, 15= Heterozygote, 16=WT.

<u>Specific Aim 1: To determine the involvement of 5-HT_{1B} heteroreceptors on GABA terminals in the VTA in the modulation of GABA release in the VTA and DA release in the ipsilateral NACC, and its involvement in the neurochemical effect of acute ethanol in freely moving animals</u>

There are two hypotheses under Specific Aim 1: one is that activation of 5-HT-1B receptors in the VTA decreases GABA release in this area and increases DA transmission in the ipsilateral NACC and the other is that activation and blockade of VTA 5-HT-1B receptors potentiates and attenuates ethanol's effects on DA transmission in the ipsilateral NACC, respectively.

The experiments under Specific Aim 1 were continued in Year 2 and the work related to Hypothesis 1 has been completed. During Year 2, we have the following findings which were not reported in the previous report.

1. Effects of infusion of CP 93129 into the VTA on extracellular DA concentrations in this region. In this experiment, dual-probe microdialysis was used. One probe was inserted into the VTA and the other in the ipsilateral NACC. Both probes were perfused with artificial cerebrospinal fluid (ACSF). After basal DA in the VTA and NACC was stable, ACSF alone and ACSF containing three different

concentrations of CP 93129 (20, 40, and 80 μM), a selective 5-HT-1B receptor agonist[2,3], were infused respectively into the VTA of separate groups of rats for 60 min. The dialysates from the VTA and NACC were collected at 20 min of intervals, and assayed via the HPLC-EC (high performance liquid chromatography coupled with electrochemical detection) system for DA in the NACC (please see the report of Year 1 for this part of results), and gamma-aminobutyric acid (GABA) and DA in the VTA (The VTA dialysates were divided into two portions: one for GABA assay and the other for DA measurement).

It can be seen from Fig 2, switching between syringes containing ACSF had no significant effects on the dialysate DA levels from the VTA. Perfusion with 20 μM of CP 93129 caused DA concentrations to increase to 127-137% of baseline but these effects did not reach statistically significant levels compared with the ACSF alone group. Perfusion with higher concentrations of CP 93129 (40 and 80 μM) produced significant increases in extracellular DA concentrations in the VTA. The maximum increases of DA levels produced by 40 and 80 μM were 182% and 242% of baseline, respectively. These results, together with those obtained during Year 1 showing increases of DA in the ipsilateral NACC following infusion of CP 93129 into the VTA (please see the report of Year 1), suggest that activation of VTA 5-HT-1B receptors by focally applied CP 93129 may increase the activity of VTA DA neurons.

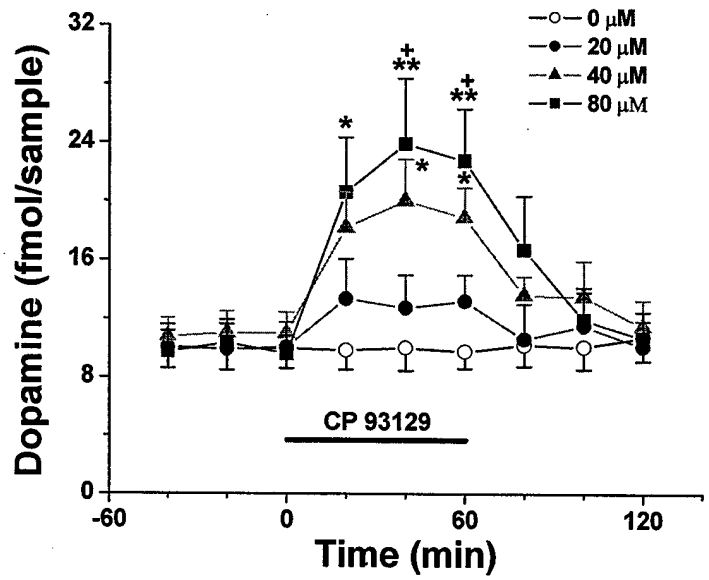

Fig 2. Effects of infusion of CP 93129 into the VTA on DA concentrations in the dialysates from this region. CP 93129 (20, 40, and 80 μM) was administered through a probe into the VTA indicated by the bar. Results are mean ± S.E.M from 6-7 animals. * $P < 0.05$, ** $P < 0.01$ as compared with the control group (0 μM); + $P < 0.05$ as compared with the 20 μM group (a two-way ANOVA followed by Tukey tests).

2. Effects of infusion of CP 93129 into the VTA on extracellular GABA concentrations in this region. As shown in Fig 3, infusion of CP 93129 at the concentration of 80 μM caused VTA GABA to decrease significantly (to 63-68% of baseline). Upon comparing the time course of VTA GABA with that of NACC DA and VTA DA following administration of 80 μM CP 93129, we found that changes in NACC DA and VTA DA correlated temporally with those of VTA GABA. This temporal correlation between the changes in GABA and DA concentrations suggests that changes in NACC DA and VTA DA may be associated, at least in part, with those in VTA GABA. Although there was a tendency towards reductions following administration of 20 or 40 μM, these effects did not reach statistical significance. The possible explanation for the lack of significant changes in GABA levels after 20 and 40 μM of CP 93129 is as follows: The part of GABA detected by microdialysis may originate from non-neuronal sources[6]. This part of GABA were not regulated by 5-HT-1B receptors located on GABA terminals (heteroreceptors) so that decreased GABA release from GABA neurons resulting from lower concentrations of CP 93129 may not cause detectable and significant changes in total extracellular GABA.

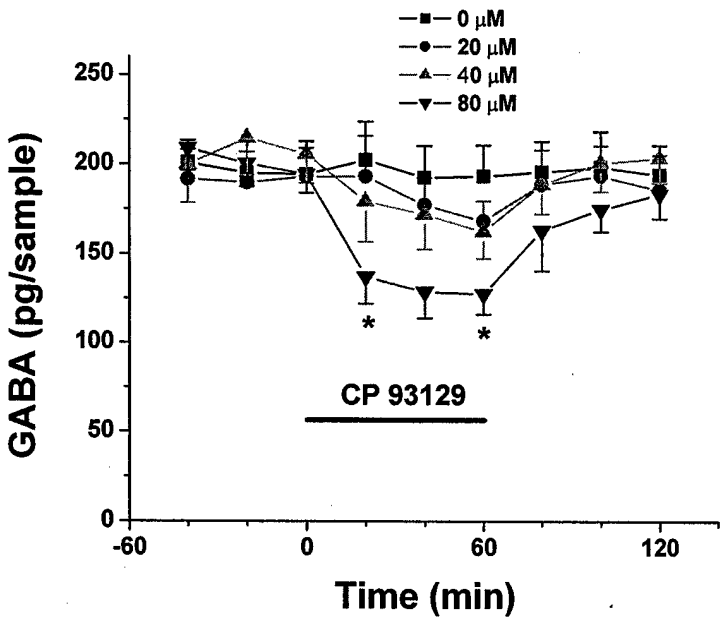

Fig 3. Effects of infusion of CP 93129 into the VTA on GABA concentrations in the dialysates from this region. CP 93129 (20, 40, and 80 μM) was administered through a probe into the VTA indicated by the bar. Results are mean ± S.E.M from 6-7 animals. * $P < 0.05$ as compared with the control group (0 μM) (a two-way ANOVA followed by Tukey tests).

3. Effects of 5-HT receptor antagonists on CP 93129-induced increases of NACC DA and VTA DA. In order to further assess the involvement of VTA 5-HT-1B receptors in the effects of CP 93129, the

experiments with 5-HT receptor antagonists were conducted during this period. The same as above, dual-probe microdialysis was used with one probe within the VTA for drug administration and the second one in the ipsilateral NACC. The following antagonists were used: SB-216641, a 5-HT-1B receptor antagonist[4], BRL-15572, a 5-HT-1D/1A receptor antagonist[4], and WAY-100635, a 5-HT-1A receptor antagonist[5]. The affinities (given as pK*i*) of these antagonists and CP 93129 for related 5-HT-1 receptor subtypes are listed in the following table. All these antagonists were infused into the VTA at the concentration of 10 μM in the perfusate for 40 min, then co-infused with CP 93129 (80 μM) for another 60 min. Dialysates from both the VTA and ipsilateral NACC were collected for assay. The dialysates from the VTA were divided into two portions: one for DA measurement and the other for GABA determination.

Compound	Receptor subtype (pK*i*)		
	5-HT-1A	5-HT-1B	5-HT-1D
CP 93129	5.7	7.8	6.0
SB-216641	6.3	9.0	7.6
BRL-15572	7.7	6.1	7.9
WAY-100635	8.9	< 6	< 6

In separate groups of rats, infusion of SB-216641 (10 μM), BRL-15572 (10 μM), or WAY-100635 (10 μM) into the VTA for 2 h showed no significant effects on extracellular DA concentrations in the VTA or NACC. As shown in Figs 2 and 3, administration of SB-216641, but not BRL-15572 or WAY-100635, blocked the ability of intra-tegmental CP 93129 (80 μM) to increase extracellular DA in the VTA and ipsilateral NACC, respectively.

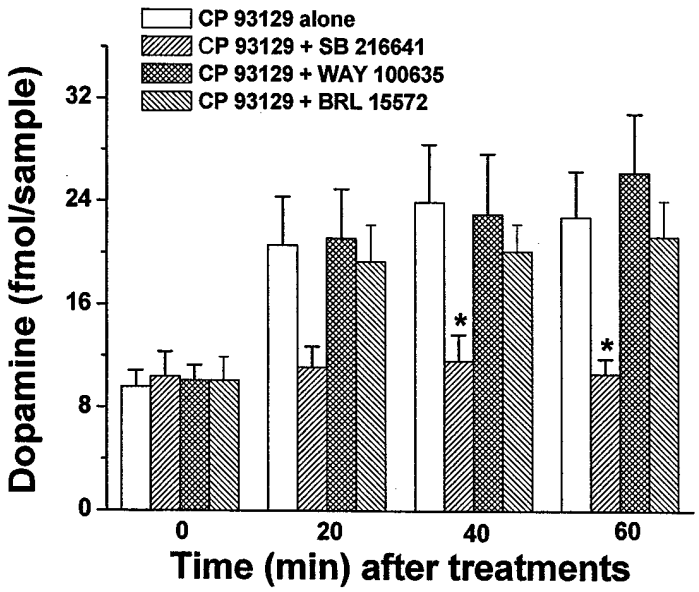

Fig 4. Effects of SB-216641 (10 μM), BRL-15572 (10 μM), and WAY-100635 (10 μM) on intra-tegmental CP 93129 (80 μM)-induced increases of extracellular concentrations of DA in the VTA.

Results are mean ± S.E.M. from 6-7 animals. * $P < 0.05$ as compared with the CP 93129 alone group (a two-way ANOVA followed by Tukey tests).

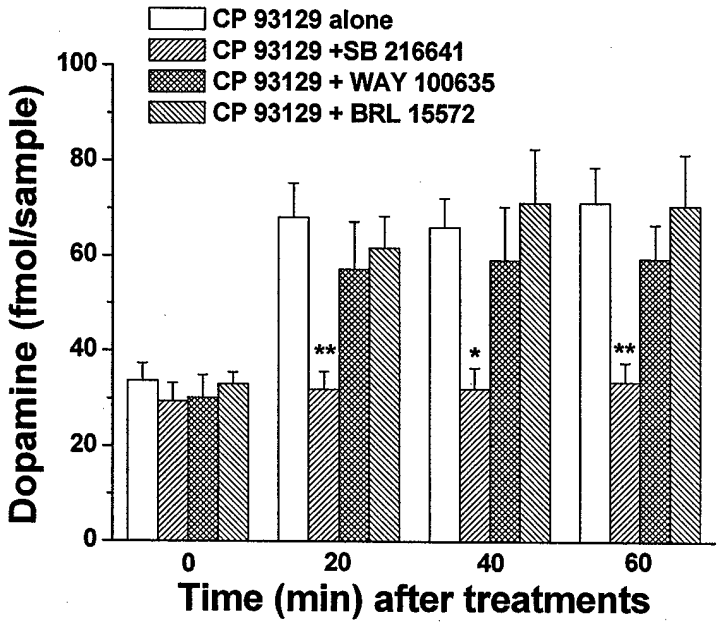

Fig 5. Effects of SB-216641 (10 μM), BRL-15572 (10 μM), and WAY-100635 (10 μM) on intra-tegmental CP 93129 (80 μM)-induced increases of extracellular concentration of DA in the ipsilateral NACC. Results are mean ± S.E.M. from 6-7 animals. * $P < 0.05$, ** $P < 0.01$ as compared with the CP 93129 alone group (a two-way ANOVA followed by Tukey tests).

4. Effects of 5-HT receptor antagonists on CP 93129-induced reductions of VTA GABA. Infusion of SB-216641 (10 μM), BRL-15572 (10 μM), or WAY-100635 (10 μM) into the VTA for 2 h showed no significant effects on extracellular GABA concentrations in the VTA in separate groups of rats. As shown in Fig 6, administration of SB-216641, but not BRL-15572 or WAY-100635, blocked the ability of intra-tegmental CP 93129 (80 μM) to decrease VTA GABA.

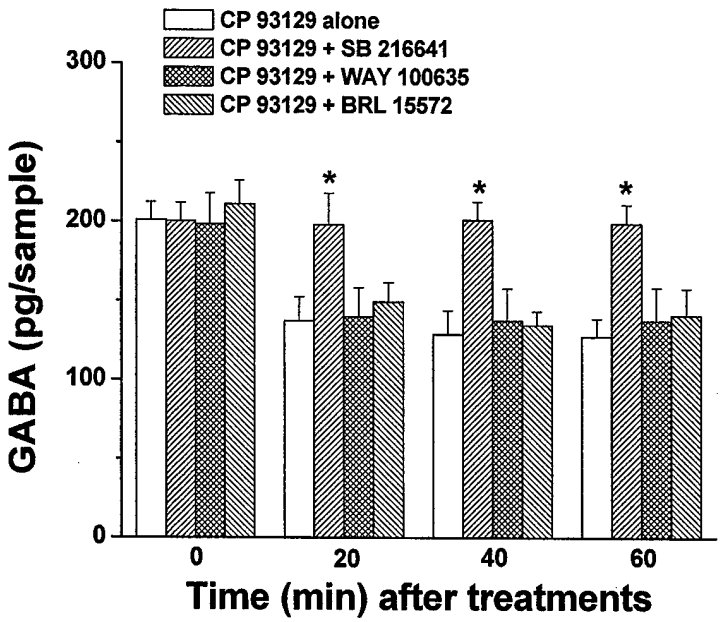

Fig 6. Effects of SB-216641 (10 μM), BRL-15572 (10 μM), and WAY-100635 (10 μM) on intra-tegmental CP 93129 (80 μM)-induced reductions of extracellular concentration of GABA in the VTA. Results are mean ± S.E.M. from 6-7 animals. * $P < 0.05$ as compared with the CP 93129 alone group (a two-way ANOVA followed by Tukey tests).

All these results will be presented as a poster at the Military Health Research Forum which will be held in San Juan, Puerto Rico, April 25-28, 2004. The abstract for this meeting is attached in the appendix. Preparation of a manuscript for publishing these data is presently under way.

KEY RESEARCH ACCOMPLISHMENTS

1. The method of genotyping of 5-HT-1B receptor knockout (KO) and their counterparts wild-type (WT) mice has been successfully established. The KO and WT mice, both of which are important subjects of experiments in Specific Aim 2, have now been generated in the PI's lab.
2. We found that administration of CP 93129, a 5-HT-1B receptor agonist, into the VTA increased DA but decreased GABA concentrations in this region. The increase of VTA DA induced by CP 93129 was concentration-dependent. The significant decrease of VTA GABA was observed only following a higher concentration of CP 93129.
3. We also observed that the effects of intra-tegmental CP 93129 on VTA DA, NACC DA and VTA GABA were all antagonized by the treatment with the 5-HT-1B receptor antagonist SB-216641 but not with the 5-HT-1D/1A receptor antagonist BRL-15572 or the 5-HT-1A receptor antagonist WAY-100635.

REPORTABLE OUTCOMES

1. Abstract for the Military Health Research Forum (San Juan, Puerto Rico, 4/25/04-4/28/04) has been attached in the appendix. A poster presentation for this meeting and a manuscript for publishing these data are presently under preparation.

CONCLUSION

The results suggest that activation of VTA 5-HT-1B receptors is associated with CP 93129-induced activation of mesolimbic DA neurons. This conclusion is based on the fact that intra-tegmental CP 93129 increased DA release in both the VTA and the NACC and that the increase of the DA release was antagonized by the 5-HT-1B receptor antagonist but not by the 5-HT-1A or 5-HT-1D receptor antagonist. The results also suggest that the 5-HT-1B receptor-mediated inhibition of VTA GABA release may contribute to the observed activation of mesolimbic DA neurons. This conclusion is drawn from the fact that intra-tegmental CP 93129 concomitantly caused a reduction of VTA GABA, an effect that was also blocked by the 5-HT-1B receptor antagonist. In addition to this indirect disinhibition of VTA DA neurons resulting from decreased GABAergic control, other mechanisms may also be involved in CP 93129-induced augmentation of mesolimbic DA transmission, particularly under lower concentrations of the drug. This speculation is based on the fact that CP 93129 increased DA release at all concentrations tested but significantly decreased VTA GABA only at a higher concentration.

There are reports in the literature showing 5-HT-1B receptor-mediated inhibition of GABA release, but most of studies were carried out on brain slices in vitro[7,8,9]. Our results provide a direct in vivo evidence for the interaction between 5-HT and DA via a VTA 5-HT-1B receptor-mediated GABA mechanism. This 5-HT-1B receptor-mediated indirect DA/5-HT interaction may be involved in several behavioral disorders such as drug addiction. For example, intra-tegmental microinjection of the 5-HT-1B receptor agonist and antagonist has recently been reported to increase and decrease discriminative stimulus effects of cocaine[10]. The involvement of this interaction in the regulation of the neurochemical effects of ethanol will be assessed in Years 3 and 4 of the project.

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INVOLVEMENT OF 5-HT1B RECEPTORS WITHIN THE VENTRAL TEGMENTAL AREA IN REGULATION OF MESOLIMBIC DOPAMINERGIC TRANSMISSION VIA GABA MECHANISMS.

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BACKGROUND/PURPOSE: This study was designed to assess the involvement of 5-HT1B receptors within the ventral tegmental area (VTA) in regulation of mesolimbic dopaminergic transmission. **METHODS:** Dual-probe microdialysis was performed in freely moving adult Sprague-Dawley rats with one probe within the ventral tegmental area (VTA) and the other within the ipsilateral nucleus accumbens (NAC). Drugs were administered into the VTA via retrograde dialysis. Dialysates from both the VTA and the NAC were collected for determination of dopamine (DA) and gamma-aminobutyric acid (GABA) by high performance liquid chromatography with electrochemical detection. **RESULTS:** Intra-tegmental infusion of CP 93129 (20, 40, and 80 μM), a 5-HT1B receptor agonist, increased extracellular DA concentrations in a concentration-dependent manner not only in the NAC but also in the VTA, indicating increased mesolimbic DA neuron activity. Administration of CP 93129 at 80 μM, but not 20 or 40 μM, into the VTA also significantly decreased extracellular GABA concentrations in this region. Co-infusion of the 5-HT1B receptor antagonist SB 216641 (10 μM), but not the 5-HT1A receptor antagonist WAY 100635 (10 μM) or the 5-HT1D receptor antagonist BRL 15572 (10 μM), antagonized not only the effects of intra-tegmental CP 93129 (80 μM) on VTA DA and NAC DA but also on VTA GABA. **CONCLUSION:** The results suggest that activation of VTA 5-HT1B receptors increases mesolimbic DA neuron activities. The increased DA neuron activity may be associated, at least in part, with the 5-HT1B receptor-mediated inhibition of VTA GABA release.

APPENDIX