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Does Electroacupuncture Affect Ethanol Modulation of Mesolimbic Neurons?

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ABSTRACT

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The purpose of this project was to investigate the mechanism of action of acupuncture on a critical neural substrate involved in alcoholism. Specifically, this study evaluated the effects of stimulation of the acupuncture Shenmen (HT7) point on inhibitory GABA neurons in the ventral tegmental area (VTA), a midbrain structure implicated in drug and alcohol abuse, and ethanol self-administration. In addition, the role of opioid receptors (ORs) in ethanol and acupuncture effects was explored. Using electrophysiological methods in mature rats, we evaluated the effects of HT7 stimulation and opioid antagonists on the VTA GABA neuron firing rate. With behavioral paradigms, we also assessed those effects on ethanol self-administration, using a modification of the sucrose fading procedure. We found that HT7 stimulation produced a biphasic modulation of VTA GABA neuron firing rate characterized by transient enhancement at the onset of stimulation followed by a prolonged inhibition and subsequent recovery in 5 min. HT7 stimulation blocked the typical suppression of VTA GABA neuron firing rate produced by a moderately intoxicating dose of ethanol. The late inhibition produced by HT7 stimulation as well as HT7 reversal of ethanol's effects on GABA neuron firing rate was blocked by the non-selective opioid receptor antagonist, naloxoneIn addition, HT7 acupuncture reduced ethanol self-administration without affecting sucrose consumption. More important, systemic administration of the δ-opioid receptor (DOR) antagonist, naltrindole blocked ethanol suppression of VTA GABA neuron firing rate and significantly reduced ethanol self-administration without affecting sucrose consumption. These findings suggest that DOR-mediated opioid modulation of VTA GABA neurons may be related to the role of acupuncture in modulating mesolimbic DA release and suppressing the reinforcing effects of ethanol. We confirmed that acupuncture stimulation may have a significant impact on the inhibitory neuron activity in the VTA and that acupuncture may serve as an effective adjunct to OR antagonist therapy for alcoholism.

Keywords: GABA, opioid, VTA, ethanol, acupuncture, dopamine, nucleus accumbens.

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Substance abuse, in general, including alcohol, tobacco, and illicit drug, is one of the most pressing health issues today. Alcohol is one of the most widely used drugs around the world. A vast conglomeration of research from various laboratories across the world has been concentrated on understanding and treating alcoholism. One of the main reasons is that the widespread use and abuse of this psychoactive drug, alcohol, result in billions of dollars of damage and millions of deaths each year. Specifically, alcohol addiction is an area of focus worth studying, due to its biological nature, abuse potential, and long-term relapsing effects. The rationale for this research is predicated on the belief that advancement in the knowledge of the neural substrates underlying the acute intoxicating and rewarding properties of alcohol will pave the way for effective treatment strategies for alcoholism that would save lives and resources throughout the world.

The objective of this study was to understand alcoholism from a neurobiological perspective, supposing that if we understood what neural substrates underlie alcohol's effects, we might be better equipped to design appropriate therapies and treatments for alcohol abuse. Acupuncture has been shown to be an effective treatment for analgesia. Yet, its effectiveness as a suitable therapy for drug addiction and specifically alcoholism is controversial. Thus, the purpose of this study was to evaluate the mechanism of action of acupuncture on critical neural substrates implicated in alcoholism. This study evaluated whether electroacupuncture stimulation of specific meridian sites might affect dopamine (DA) neurons in the mesolimbic system implicated in reward and addiction, DA neurotransmission in this system, and its implication over the effects of ethanol on them. I hypothesized that electroacupuncture would

affect the firing rate and discharge properties of gamma-butyric amino acid (GABA) neurons in the ventral tegmental area (VTA) as well as DA neurotransmission to the nucleus accumbens (NAcc), and that it would block the well-known effects of acute intoxicating levels of alcohol on these neural responses. In addition, the role of opioids in mediating acupuncture's effects was covered. In this thesis, I will : 1) Briefly review alcohol abuse and alcoholism, and the mesocorticolimbic DA reward system; 2) Describe the DA pharmacology of ethanol effects and the biology of electroacupuncture and its relation to alcohol addiction; 3) Summarize the research regarding VTA GABA neurons and their roles in the mesocorticolimbic DA system; 4) Present our experimental results, and 5) Discuss the implications of these results while providing an overview of critiques to help in future studies.

Background

Alcohol Abuse and Alcoholism

Approximately 14 million Americans, more than 7 percent of the entire population, meet the diagnostic criteria for alcohol abuse or alcoholism (Gordis, 2000). One in four children younger than 18 years old in the United States is reported to be exposed to alcohol abuse or alcohol dependence in the family (Grant, 2000). The economic and societal implications arising as direct or indirect result of alcohol consumption are astounding. The direct economic cost of alcohol abuse in the United States is as high as $185 billion annually (Gordis, 2000), or roughly $638 for every man, woman, and child living in the country in 1998 alone (Howard et al, 2000). The indirect costs associated with alcohol abuse are remarkably staggering as well. For example, alcohol-induced hangovers cost an estimated $148 billion in related absenteeism and poor job performance in work only (Wiese, Shlipak, & Browner, 2000). The compulsion to consume

alcohol is thought to stem primarily from its positive reinforcing properties including the anxiolytic and euphoric effects as well as its negative reinforcing properties that derive from the development of dependence, wherein alcohol is ingested to avoid the aversive consequences of abstinence (G.F. Koob, Rassnick, Heinrichs, & Weiss, 1994). Previous studies involving intravenous self- administration of opiates and psychostimulants have identified specific neural substrates that mediate the reinforcing actions of these drugs, and found that they constitute a part of the same reward system in the brain that has evolved for mediating natural motivated behaviors and the mesocorticolimbic DA system (Floyd E. Bloom, 1993; Kalivas, Churchill, & Klitenick, 1993; G.F. Koob, 1992; Schultz, Dayan, & Montague, 1997).

The Mesocorticolimbic Dopamine System and Reward

The mesocorticolimbic system is an area in the brain that is known to be involved in natural and drug reward as well as addiction. In this system, several parts of the brain including the hippocampus, amygdala, medial prefrontal cortex, nucleus accumbens and ventral tegmental area are connected by pathways (Pierce & Kumaresan, 2006). The ventral tegmental area (VTA) and nucleus accumbens (NAcc) in particular are considered to be reward centers that are affected by alcohol. Within the VTA, there are two main types of neurons: dopamine (DA) and γ-aminobutyric acid (GABA). The pathways from both of these types of neurons in the VTA are linked to other parts of the limbic system and in turn the VTA receives GABAergic input from the NAcc. DA and GABA neurons have distinctive firing patterns in anesthetized adult male rats. DA neurons fire slowly and sometimes in bursts whereas GABA neurons fire at a much higher frequency and do not fire in bursts (S. C. Steffensen, Svingos, Pickel, & Henriksen, 1998). Using this distinctive firing pattern of these neurons, we can easily distinguish between the two neurons in the brain *in vivo*.

The prevailing dogma behind reward and addiction in the brain is that an euphoric state is achieved through increased DA levels in the mesocorticolimbic system, particularly the NAcc and the VTA (Wise, 2004). Thus, much of drug and alcohol research has been dedicated to studying DA neurons in this system of the brain. Although activation of the mesocorticolimbic system is implicated in drug reward, the DA levels in the system is critical only for psychostimulant reward (G.F Koob, 1996), as rats continue to self-administer ethanol and opioids, despite severe neurotoxin-lesions of the mesolimbic DA system (G.F. Koob, 1992). Thus, this research is centered on GABA neurons in the VTA.

Ethanol Neuropharmacology

The two major types of membrane-bound proteins that are directly affected by physiologically-relevant levels of ethanol (i.e., concentrations up to 100 mM or 460 mg/dL, at which point ethanol can be lethal to humans) are ligand-gated ion channels and voltage-dependent calcium channels(R. A. Harris, 1999). There is a compelling evidence that ethanol interacts with calcium channels(Leslie, Barr, Chandler, & Farrar, 1983; Mohri, et al., 2003; Walter & Messing, 1999), M-type cholinergic receptors(Moore, Madamba, & Siggins, 1990), and G-proteins(F. Lee & Wand, 1996). At the cellular or neurochemical level, there is a confirmatory evidence to suggest that ethanol can alter GLU function(P. Hoffman, Rabe, Moses, & Tabakoff, 1989; P. L. Hoffman & Tabakoff, 1993; Lima-Landman & Albuquerque, 1989; Lovinger, White, & Weight, 1989; D. M. Lovinger, G. White, & F. F. Weight, 1990; D.M. Lovinger, G. White, & F.F. Weight, 1990; Roberto, et al., 2004), that low doses of ethanol may interact with the GABA receptor complexes(Allan & Harris, 1986; Liljenquist & Engel, 1982; Mihic & Harris, 1996; Suzdak, et al., 1986; Ticku, Lowrimor, & Lehoullier, 1986), that ethanol can interact with neuromodulators such as neurosteriods(Brot, Akwa, Purdy, Koob, & Britton,

1996; Kumar, Fleming, & Morrow, 2004; Lambert, Peters, & Cottrell, 1987; Simmonds, 1991; Wieland, Belluzzi, Stein, & Lan, 1995), and that ethanol influences several neuropeptide functions including somatostatin(G.R. Siggins, Nie, Schweitzer, Madamba, & Henriksen, 1996) and corticotropin releasing factor(G.F. Koob, Heinrichs, Menzaghi, Pich, & Britton, 1994). These observations reinforce the likelihood that behaviorally relevant doses of ethanol have their effects by altering neuronal synaptic efficacy(Sinclair, 1990).

Synaptic transmission is depressed by acute intoxicating doses of ethanol (Ariwodola, et al., 2003; Berry & Pentreath, 1980; F.E. Bloom, et al., 1984; Deitrich, Dunwiddie, Harris, & Erwin, 1989; Shefner, 1990; G. R. Siggins, et al., 1987), which might result from either an attenuation of excitatory amino acid synaptic transmission(Lovinger, et al., 1989; D.M. Lovinger, et al., 1990; Nie, Yuan, Madamba, & Siggins, 1993; Roberto, et al., 2004; White, Lovinger, & Weight, 1990) or an enhancement of inhibitory GABA transmission(Deitrich, et al., 1989; A. R. Harris & Allan, 1989; Roberto, Madamba, Moore, Tallent, & Siggins, 2003). However, the prevalent hypothesis is that the reinforcing properties of ethanol are primarily implemented by its inhibitory actions on excitatory synaptic transmission mediated by the N-methyl-D-aspartic acid (NMDA) receptor. The subunit composition of the NMDA receptor appears to be important for ethanol effects. Developmental decreases in ethanol inhibition of NMDA receptor function in cortical neurons seem to parallel incremental decreases in inhibition by ifenprodil, a selective inhibitor of NMDA receptor, in which the NR2B subunit of NMDA receptor is essential for its action(Lovinger, 1995).

Ethanol and The Mesocorticolimbic System

The existence of NAcc as a key area and its proximal connections in the mesolimbic system are affirmed with an evidence obtained from the studies demonstrating that local

injections of neurotransmitter antagonists into the NAcc and/or afferent circuit systems prevent ethanol self-administration in rats (Hyytia & Koob, 1995; G.F. Koob, 1991; G. F. Koob, Pettit, Ettenberg, & Bloom, 1984; Pettit, Ettenberg, Bloom, & Koob, 1984; Stefanie Rassnick, et al., 1991; Roberts, Cole, & Koob, 1996; Vaccarino, Bloom, & Koob, 1985). An interesting finding was that rats will self-administer ethanol directly into the VTA(Gatto, McBride, Murphy, Lumeng, & Li, 1994) and an ethanol-induced increase of DA release in the NAcc, detected by microdialysis, has been reported extensively(Imperato & DiChiara, 1986; Wozniak, Pert, Mele, & Linnoila, 1991; Yoshimoto, McBride, Lumeng, & Li, 1992) (see also(Di Chiara & Imperato, 1985, 1988; B. Weiss, 1991; Friedbert Weiss, et al., 1991)). A lack of DA involvement in drug reinforcement has also been demonstrated for oral ethanol self-administration (Kiianmaa, Andersson, & Fuxe, 1979; Myers & Quarfordt, 1991; S. Rassnick, Stinus, & Koob, 1993) and ethanol conditioned place preference (Cunningham & Noble, 1992; Risinger, Dickinson, & Cunningham, 1992). Furthermore, ethanol self-administer is blocked by intra-amygdalar injections of GABA agonists in dependent rats(Roberts, et al., 1996) and GABA antagonists in non-dependent rats (Hyytia & Koob, 1995), suggesting DA-independent mechanisms. Regarding DA neurons in the VTA, ethanol increases their firing rate both *in vivo* and *in vitro* similar to what has been observed for many other drugs of abuse (Brodie & Appel, 1998; Gysling & Wang, 1983; Mereu, K-W., Gessa, Naes, & Westfall, 1987).

Dopamine Independent Mechanism

The mesocorticolimbic DA system originating in the VTA and projecting to the NAcc is known to be involved in the reward from natural behaviors such as feeding (Ahn & Phillips, 2002, 2003; Phillips, Ahn, & Howland, 2003), drinking (Agmo, Federman, Navarro, Padua, & Velazquez, 1993; Agmo, Galvan, & Talamantes, 1995), and other types of rewards such as

intracranial self-stimulation (ICSS) (Gratton & Wise, 1983; Tzschentke, 2000; Wise, 2002). The mesocorticolimbic DA system has also been implicated in the habit-forming actions of several addictive drugs (Wise, 2004). Early versions of hypothesis for DA reward suggested that DA might be crucial for all kinds of drug reward, but phencyclidine, morphine, and nicotine had both DA-dependent and DA-independent rewarding effects. The emerging view is that DA is crucial for the rewarding effects of the psychomotor stimulants and is important, but may not be critical for those of the opiates, nicotine, cannabis and ethanol. In support of DA-independent mechanisms for reward, a considerable number of electrophysiological studies in freely-moving animals have shown that only a low percentage of NAcc neurons exhibit discharge correlations either during heroin, cocaine, or ethanol self-administration, or focused attention (Carelli & Deadwyler, 1994; Chang, Zhang, Janak, & Woodward, 1997; Peoples & West, 1996). The fact that DA is less involved in drug reinforcement has also been demonstrated for cocaine self-administration (Goeders & Smith, 1983) and cocaine CPP(Mackey & Van der Kooy, 1985; Spyraki, Fibiger, & Phillips, 1982). Besides, the role of DA in cocaine self-administration has been called into question by studies demonstrating that DA-transporter knock-out mice continue to consume cocaine(Rocha, et al., 1998).

In addition , the relation of DA to ICSS has also been demonstrated (Kilpatrick, Rooney, Michael, & Wightman, 2000). Consistent with the lack of DA engagement, repeated administration of NMDA antagonists into the VTA does not seem to produce behavioral sensitization (Cornish, Nakamura, & Kalivas, 2001). Furthermore, the accumulating evidence strongly suggests that GABA neurons in both the VTA and the NAcc appear to play significant roles in opioid reward (Xi & Stein, 2002). More recently, it has been reported that $GABA_A$ receptors in the mammalian VTA serve as a mediator for potential addiction switching

mechanism by gating reward transmission through one of the two neural motivational systems: Either a DA-independent (opiate-naive) or a DA-dependent (opiate-dependent or opiate-withdrawn) system(Laviolette, Gallegos, Henriksen, & van der Kooy, 2004). After opiate exposure and its subsequent withdrawal, the functional conductance properties of the VTA $GABA_A$ receptor switch from an inhibitory to an excitatory signaling mode. Other behavioral studies have shown that chemical destruction of DA terminals in the NAcc with the DA neurotoxin 6-hydroxydopamine has no effect on morphine or heroin self-administration (Dworkin, Guerin, Co, Goeders, & Smith, 1988; Ettenberg, Pettit, Bloom, & Koob, 1982; Pettit, et al., 1984).

These findings challenge the notion at least for opioids, that the DA circuits are essential for drug-seeking behavior(DiChiara & North, 1992; Fontana, Post, & Pert, 1993; Robinson & Berridge, 1993). Indeed, it has been suggested that DA neurons do not appear to be reward neurons *per se*, but may be critical for initiating drug use. More importantly, they may be useful in reinstating drug use during protracted abstinence(G. F. Koob & Le Moal, 1997). Consequently, DA neurotransmission may be important only in mediating the motivational effects of drugs in dependent animals and that the other areas of substrates in the brain mediate the rewarding properties of drugs when animals are non-dependent(Bechara & van der Kooy, 1992; Nader & van der Kooy, 1994).

GABA Neurons in The Mesocorticolimbic System

GABA neurons synapse on DA neurons in the VTA, thereby providing an inhibitory input to regulate DA release. This is in agreement with the previous finding of GABAergic control over DA neurons in the substantia nigra (Tepper, Paladini, & Celada, 1998). Inhibition of these inhibitory GABA neurons would result in hyperexcitability of DA neurons and an

increased amount of DA release. Previously, it has been shown that alcohol decreases the firing rate of these GABA neurons with acute administration for up to 2 weeks (Gallegos, Criado, Lee, Henriksen, & Steffensen, 1999). In line with our studies and the DA theory, the decreased firing rate of the GABA neurons would result in more DA neurons to be released and a euphoric state would be followed. After 2 weeks of daily alcohol administration, however, the firing rate no longer decreases (Gallegos, et al., 1999). This indicates that some sort of tolerance occurs with these GABA neurons toward ethanol, resulting in hyperactivity of the GABA neurons. As a result, decrease in DA release is followed, which leads to a withdrawal state after alcohol is discontinued.

Another interesting phenomenon we have found in the lab is that high-frequency stimulation of internal capsule (IC) in the brain causes multiple post-stimulus spike discharge (ICPSDs) of GABA neurons in the VTA (S. C. Steffensen, et al., 1998). These discharges are blocked by gap junction (GJ) antagonists, suggesting that VTA GABA neurons are part of a network connected electrically by GJs (S.C. Steffensen, et al., 2003). In addition, we have shown that acute ethanol administration also suppresses VTA GABA neuron ICPSDs, with an IC_{50} at a dose of 1.1 g/kg of ethanol (Stobbs, et al., 2004), a moderately intoxicating dose. We have recently studied VTA GABA neuron firing rate and ICPSDs during chronic ethanol consumption as well. In this study, we have found that neither the firing rate of GABA neuron nor ICPSDs adapt to chronic ethanol consumption. This was somewhat surprising to us as VTA GABA neuron firing rate adapts to chronic ethanol injections. The disparity between two different conditions may lie in the fact that rats do not become dependent on ethanol in the consumption paradigm, but they are dependent on ethanol in the forced injection paradigm. Thus, it may require dependence on a reinforcer to exhibit physiological adaptation.

Nonetheless, in line with this study, we did find that DA D2 receptor expression adapts to chronic ethanol consumption.

Dopamine, as we have seen thus far, plays an important role in the mesocorticolimbic system. Acute and local administration of DA activates GABA neurons, increasing their firing rate of GABA neurons up to 100-200% (Stobbs, et al., 2004). Recently, this activation has been shown to occur through the D2 receptor, given that D2 receptor antagonists block the activation. As acute ethanol decreases the firing rate of VTA GABA neurons but chronic ethanol increases their firing rate, it would seem logical to look at expression levels of this protein to see if they are also affected by chronic ethanol.

Acupuncture

The acupuncture, believed to be effective to attenuate pain from trauma, was known in *The Nei Ching* or *The Yellow Emperor's Classic of Internal Medicine* in China (S. T. Chang, 1976). Acupuncture is defined as "insertion of a solid needle into the body for the purpose of therapy, disease prevention or maintenance of health" ((T.M., 2006), p. 3). Inevitably, acupuncture should include a needle and skin penetration as traced from its meaning in Latin, *acus* meaning needle and *puncture* referring to penetration. And acupuncture may cause several local traumas in the skin where it is used, due to its sharp-pointed tip of the needle.

In the past, Chinese people believed that humans are capable of restoring equilibrium in the body under any circumstances, even after being disturbed from internal or external events, and thought that some kind of life force by which they can maintain a perfect balance is inherent in the body. Recorded in the *Tao Te King* (circa 200 -100 B.C.), Tao is described as the abstract force responsible for creating, changing, and developing all things in nature (T.M., 2006). In Traditional Chinese Medicine (TCM), there are two important dualities that always exist in

nature derived from the Tao, *Yin* and *Yang*. While Yin refers to the shady side, Yang represents the sunny side of the hill in the old Chinese ideogram. The opposites complement each other in a dynamic process. Thus, they are never imagined to stand alone without the other, contributing to making the whole. *Yin* condition is summarized as receptive, cold, and deficient whereas *Yang* is explained as the condition of creative, hot, and excessive (Gabriel Stux, 1991). Tao, creative force, is believed to give rise to *the flow of life force*, called "Qi", which is apparent in all life in the form of change and movement, and is omnipresent in nature. Stux (1991) argued that the metaphysical concept, Qi flows in channels or meridians to perform several main functions: initiation of voluntary and involuntary movements, generation of warmth in the body, and protection of body from external noxious influences. But when the Qi is disturbed or blocked, then disease occurs, bringing about imbalance in the life force. By inserting needles into specific points along 12 meridians of the body, acupuncture is considered to alter the flow so that Qi can be restored. Chang (1976) maintained that some Chinese physicians had already found the fact that there was a pathway for nerve impulses generated in response to both internal and external stimuli, and discovered the correlation between meridians and nerve pathways. Yet, it is still unclear how they are specifically related to each other. The TCM concepts describe every part of the body as being connected by meridians on which the acupuncture points are located and most of them are linked to the vital organs of the body. The meridian system may be thought of as an invisible and intangible network along which messages are sent from acupuncture points to various parts of the body.

Particularly, the messages sent by insertion of needles into acupuncture points may either enhance or reduce activity of Qi, depending on how and which specific acupuncture points are excited. In addition, the way in which the needle is inserted might have influence on whether it

would be either sedation or activation (Wensel, 1980). Furthermore, it is surprising that acupuncture may contribute to facilitate electrochemical information transfer between synapses in the neural circuit, due to its repetitive activity and stimulation. And it appears that acupuncture may enhance the biochemical balance in the central nervous system and maintenance or recovery of homeostasis (M. R. Kim, et al., 2005; C. H. Zhao, Stillman, & Rozen, 2005). Besides, some results of animal studies regarding drug addiction have provided evidence for involvement of neurotransmitters in the action of acupuncture (Otto, 2003; Yang, Lee, & Sohn, 2008). Since its introduction to the West, acupuncture has been useful in relieving pain or disabilities after physical surgery, lessening the symptoms of neurologic disorders, or even treating for chronic allergies effectively. Especially, both electroacupuncture and Transcutaneous Electric Stimulation (TES) have proven so successful that they might be likely to be of use for relief of depression or mental illness as well. Some studies in the United States showed that many of deaths in operation were caused by anesthesia rather than surgery. Also, many kinds of the drugs used in hospitals were reported to have unfavorable side effects as well as addiction. On the contrary, acupuncture is considered safer than anesthetic drugs in that it doesn't bring about allergic reaction from patients or have undesirable side effects, which may decrease the quantity of required drugs for surgery in medical field.

Electroacupuncture, a modern phenomenon, was recently developed in China when manual acupuncture was still being used to induce analgesia for surgery in the hospital with a view to reducing the number of acupuncturists who were constantly stimulating in the procedure. With attached flexible wires to the needles, this new type of acupuncture began to provide electricity as a source for stimulation. After several years of clinical practice, the electroacupuncture was formally applied to the treatment of chronic pain and neurological

disease in the late 1960s. Although both electroacupuncture and TES were found to be somewhat correlated in implementing, when compared the analgesic effect induced by types of stimulation, electroacupuncture showed a greater effect than TES or manual acupuncture, (Ulett, Han, & Han, 1998). Generally, both sufficient voltage to overcome resistance of tissues and adequate amount of current to depolarize the nerves are required in implementing electroacupuncture. Because of the difference in frequency that stimulates the neurotransmitters involved in the therapeutic effects of acupuncture, a range of frequencies, 2 to 100Hz, instead of specific frequency is applied to release as many neurotransmitters as possible in accordance with individual purpose. Today, it seems acceptable that electroacupuncture is more productive than manual acupuncture because the former appears to produce a greater range of related neurotransmitters than the latter. In treatment of chronic pain, the electroacupuncture has shown to have a longer-lasting effect than manual needling. Moreover, it seems that a high frequency electroacupuncture may have a particular application in treatment of drug withdrawal symptoms (T.M., 2006).

Acupuncture and Addiction

So complex is the addiction to the drug of abuse that no specific treatment could be easily suggested as an ultimate solution. Wensel (1980) described that addiction is a state in which obsessive craving for excessive amounts of a substance is repeated. She also pointed out that in most cases, addiction may involve not only physiologic problems but also psychological characteristics at the same time. Although equally dangerous to withdraw from drugs suddenly whatever they may be, it may be said that withdrawal symptom from alcoholism is regarded as most fatal. Alcohol is assumed to be the most readily available tranquilizer for relieving pain. However, its potential as a killer is not known as widely as expected. Consequently, it is almost

impossible for alcoholics to keep away from alcohol for a day or two without intensive medical assistance. With its unique characteristic, acupuncture has greatly contributed to eliminating self-destructive addictions by suppressing the agitated energy flow within the body. Balancing the energy is believed to replace the desire for alcohol by regulating metabolism in the body and giving a feeling of well-being which would make it easier to give up the drug (D. S. T. Chang, 1976).

Empirically, it is shown that the acupuncture points on the heart meridians have a sedative effect, and the specific ear points play a role in alleviating the withdrawal symptoms from alcohol (Gabriel Stux, 1991). And Ulett et al (1998) reported that clinical studies on heroin addicts showed that the treatment with electroacupuncture significantly decreased heart rate and produced the sensation of well-being, while it attenuated the heroin withdrawal syndrome. However, some studies showed that the use of transcutaneous stimulation of acupuncture points with a Han's acupoint and nerve stimulator (HANS) at identified frequencies, 2 to 100 Hz, produced the most significant improvement on the opioid withdrawl syndrome for heroin addicts (Han, Chen, Yuan, & Yan, 1994). In addition, according to Chu et al (2007), chronic morphine administration induced cell size reduction of VTA DAergic neurons in rats, which could be reversed by the specific frequency electroacupuncture treatments. She added that this finding suggests new evidence that electroacupuncture may serve as a potential therapy in treating opiate addiction. It looks obvious that acupuncture is effective for the treatment of post-operative and chemo-therapy induced nausea or vomiting as well as alcohol dependence. But, in reality whether acupuncture is effective on addiction or not seems still controversial. A meta-analysis study where 22 controlled clinical studies were evaluated for the assessment of acupuncture effect on addiction showed no solid results (Brewington, Smith, & Lipton, 1994). Especially, for

alcohol dependence, only a small number of coherent studies have been conducted. Thus, there are still other remaining medical conditions in which effectiveness of acupuncture has not been proposed with strong evidence yet. It could be one of the reasons that acupuncture has still remained as an adjunct treatment or acceptable alternative in clinical fields in western countries. With regard to the effect of acupuncture on treating various addictions, research on its clinical utility has been somewhat negative. In part, because of methodological inadequacies in the studies, these studies have been regarded as inappropriate for replication. Thus, it seems unlikely that acupuncture would be accepted into the West as a useful clinical modality until a more strict research design method using adequate sample size, truly double-blind, or sham-controlled experiment is satisfied (Mayer, 2000). But in spite of the fact that little is known about the basic mechanism of acupuncture in treating drug addiction, neurochemical and behavioral data have consistently demonstrated that acupuncture directly or directly has a clear impact on the mesolimbic dopamine system (Yang, et al., 2008).

Alcohol, an addictive drug, has long been implicated in the risk of bringing about stroke-induced brain damage and can result in serious cognitive and neurological disorder. Particularly, alcohol- induced euphoria may be linked to rapid increase in DA release in limbic areas of the brain (Yoshimoto K, 1991). In his experiment with rats challenged with restriction and stress, Yoshimoto (1991) suggested that stress or anxiety may stimulate voluntary alcohol consumption in animal models. But with the help of electroacupuncture on specific sites, ST 36 (Tsu-San Li), he demonstrated that the alcohol-drinking behavior could be significantly reduced. However, ST 36 acupoint, a major point of the stomach channel, is known for its suppressing effect on morphine or heroine withdrawl syndrome rather than alcohol (Wu, Cui, Tian, Ji, & Han, 1999).In the course of exploring the basic mechanism of acupuncture in the treatment of drug abuse, they

found that GABAergic neurons dampen DA neurons via inhibitory $GABA_B$ receptors in the mesolimbic DA system (Yoon, et al., 2004). In their experiment with rats administered with alcohol, acupuncture was given at HT7 (Shenmen) points (see Figure 1), which has been clinically used to treat mental disorder and drug abuse in oriental medicine. Acupuncture at HT7 on heart channel significantly decreased DA release in NAcc in response to alcohol. It appears that acupuncture at HT7 may have a strong inhibitory effect on alcohol-induced DA release in the NAcc by activating $GABA_B$ receptors. Numerous studies have shown that chronic administration of alcohol may produce depletion or sensitization of extracellular dopamine levels in the NAcc. In other chronic experiments, rats were injected with either alcohol or saline for 21 days. Following 3 days of alcohol withdrawal, acupuncture was applied to HT7 in different-conditioned rats (R. J. Zhao, et al., 2006). They suggested that stimulation on HT7 by acupuncture significantly prevented decrease of extracellular DA levels in the NAcc during alcohol withdrawal period and blocked increase DA levels induced by alcohol at the same time. Therefore, it should be suggested that stimulation of the specific site on acupuncture points, HT7 seems to normalize the release of DA in the mesolimbic system following chronic alcohol administration.

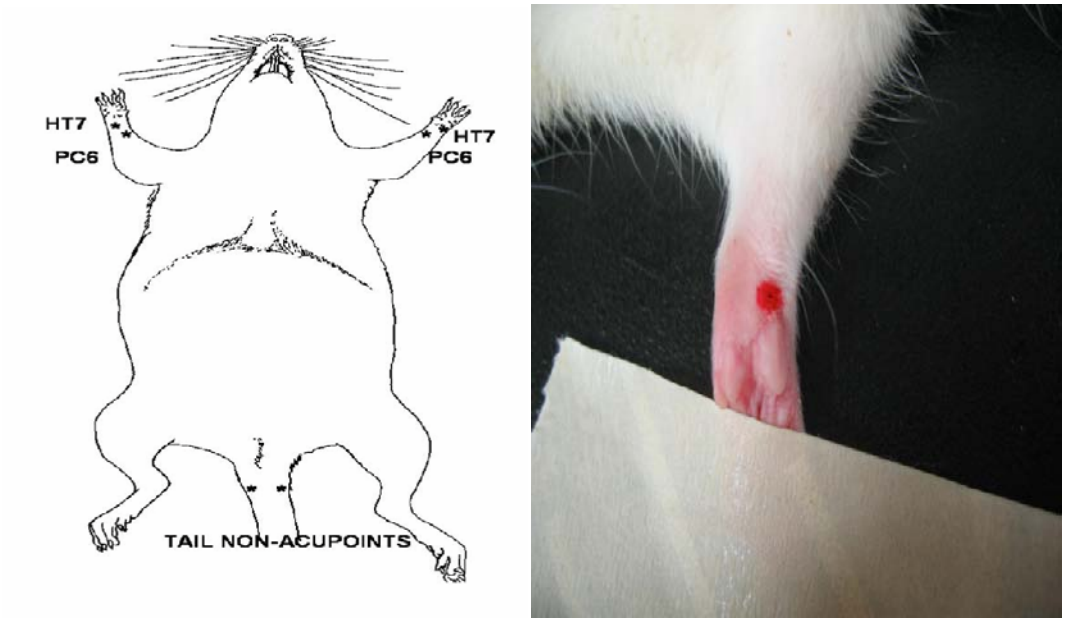


Figure 1. **Stimulation of acupuncture sites.** Acupuncture Shenmen (HT7) points were stimulated mechanically for alcohol self-administration studies or stimulated electrically (2 Hz at threshold current for muscle twitch) for single-unit electrophysiology studies. It shows the locations of HT7, PC6, and Tail acupuncture sites.

Withdrawal from chronic alcohol administration has shown that it can cause DA level to decrease in the NAcc and it leads to withdrawal signs including tremor or hyper-motility that contributes to intense alcohol craving of addicts (B. H. Lee, et al., 2008). In order to understand better the mechanism underlying a normalizing effect of acupuncture, investigation on the potential role of GABA receptors was performed. Alcohol was administered and those GABA receptor antagonists in the mesolimbic system were applied before HT7 acupuncture. According to Yang (2006), $GABA_B$ receptor antagonist reversed the suppressive effect of acupuncture on DA depletion in NAcc. In contrast, $GABA_A$ receptor antagonist significantly blocked acupuncture's suppression of alcohol withdrawal symptoms without affecting inhibitory effect of acupuncture on DA level depletion in the NAcc. In brief, GABA receptors may be involved in

acupuncture's role in normalizing DA release during and after the chronic alcohol challenge. In other words, acupuncture at the specific acupoint, HT7 prevented a reduction of DA release in the NAcc during ethanol withdrawl and inhibited an increase ethanol-induced DA levels in the same NAcc during its administration (R. J. Zhao, et al., 2006).

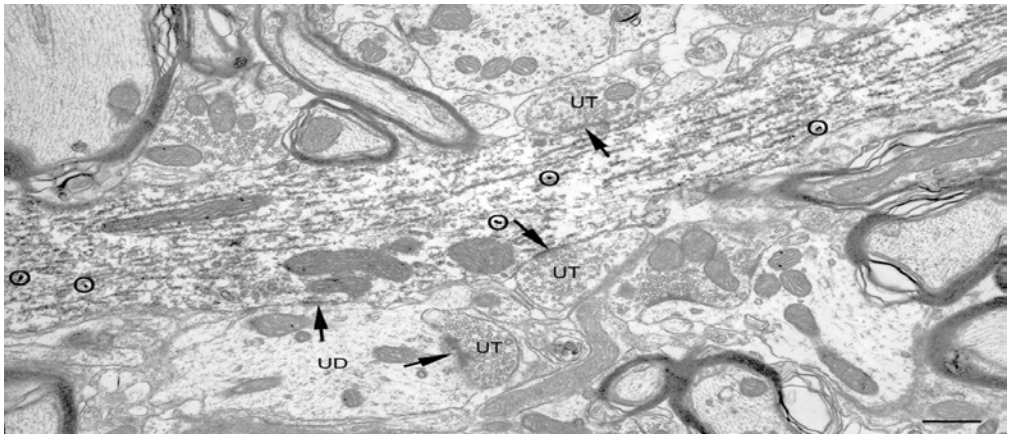


Figure 2. **μ-opioid receptor(MOR)s are localized to VTA GABA neurons**. The electron micrograph shows a longitudinally-cut dendrite containing peroxidase reaction product for neurobiotin, indicative of a physiologically characterized neuron. The neurobiotin-filled dendrite contains immunogold-silver particles for MOR (circles) that are localized to intracellular organelles. Two unlabeled terminals (UT) form asymmetric-type synapses (arrows) with the dually-labeled dendrite. In addition, an unlabeled dendrite (UD) forms a tight junction with the neurobiotin/MOR dendrite (arrow). Scale bar, 1.0 μm.

Hypotheses

The neurobiological substrate for acupuncture analgesia is believed to involve endorphinergic neurons in the arcuate nucleus of hypothalamus (Yu & Han, 1989), which project to the VTA and the NAcc (Mansour, Khachaturian, Lewis, Akil, & Watson, 1988). Electroacupuncture has been shown to activate endogenous opioidergic neurons in the arcuate nucleus of hypothalamus (Q. Wang, Mao, & Han, 1990a, 1990b). Thus, this neurochemical interaction lends support to the notion that endogenous opioids released by acupuncture may stimulate MORs on GABA neurons in the VTA (S. C. Steffensen, et al., 2006). Activation of MORs by endogenous opioids or exogenous opiates hyperpolarizes these GABA neurons, resulting in inhibition of their activity (S. C. Steffensen, et al., 2006), disinhibition of DA neurons, consequently leading to an increase in NAcc DA release (S. W. Johnson & R. A. North, 1992).

An interesting fining was that MORs are involved in acupuncture's role in suppressing morphine-induced place preference in rats (B. Wang, Luo, Xia, & Han, 2000). Also, it has been shown that electroacupuncture inhibits voluntary intake of ethanol in alcohol-preferring rats and this inhibitory effect is reversed by naltrexone, non-selective MOR antagoinist (Overstreet, et al., 2008) (see Table 1). As suggested by many studies, acupuncture has shown to reduce alcohol-drinking behaviors in rats (Yoshimoto, et al., 2001) by increasing striatal DA level and decreasing withdrawal signs in ethanol-withdrawn rats (J. H. Kim, et al., 2005). Importantly, our previous microdialysis study demonstrated dual paradoxical effects that acupuncture at Shenmen (HT7) points significantly inhibited a reduction of extracellular DA levels in the NAcc during ethanol withdrawal, and suppressed an enhancement in NAcc DA levels induced by the ethanol challenge (R. J. Zhao, et al., 2006).

Table 1

Opioid receptor-ligands relationship

Receptor type	Endogenous ligands	Exogenous ligands	Antagonists	
			Non-selective	Selective
Mu	β- endorphin	Morphine		CTOP
Delta	Enkephalins	DPDPE		ICI 174,864
		Deltrophin	Naloxone[a]	Naltrindole[b]
			Naltrexone	β – Funaltrexamine
			Nalmefene	Naltriben
Kappa	Dynorphins	U 50488		
		U 69593		Nor-binaltorphimine
		E 2078		

Note. [a] a non-selective antagonist. [b] a specific selective antagonist.

One study showed that the reduced ethanol-reinforced behavior produced by the opioid receptor antagonist, naltrexone is mediated via the parallel decrease of extracellular DA levels in the NAcc (Gonzales & Weiss, 1998). On the basis of these findings, it appeared that acupuncture might affect VTA GABA neuron excitability and suppress ethanol self-administration behavior through MORs on GABA neurons In the VTA. The goal of this project was to evaluate the

effects of electroacupuncture on GABA neurons, DA release in the NAcc, and acute and chronic ethanol effects on DA neurotransmission in the NAcc. Therefore, we evaluated the effects of electroacupuncture on VTA GABA neuron firing rate, the ability of ethanol to markedly suppress the firing rate of VTA GABA neurons, and its ability to increase release of DA in the NAcc. Since VTA GABA neurons express MORs, I hypothesized that stimulation of specific acupuncture points would modulate the activity of VTA GABA neurons, and that MOR antagonists would block the modulation by acupuncture as well as ethanol effects on VTA GABA neurons.

Methods

Animals and Surgical Procedure

Male Sprague-Dawley rats which were obtained from Charles River Laboratory (Hollister, CA) were bred and cared for in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were housed two in a cage from the time of weaning with *ad libitum* access to food and water. The room temperature was controlled (22ºC to 25ºC) and maintained on a reverse 12 hr light/dark cycle (OFF 08:00 hrs, ON 20:00 hrs). Animal care, maintenance and experimental procedures were in accordance with the Brigham Young University Animal Research Committee and met or exceeded National Institutes of Health guidelines for the care and use of laboratory animals.

For acute electrophysiological recordings of VTA GABA neurons, the rats were anesthetized using isoflourane and placed in a stereotaxic apparatus. Anesthesia level was maintained at 1% throughout the experiments. Body temperature was maintained at 37.4 ± 0.1ºC

by a feedback regulated heating pad. With the skull exposed, holes were drilled for placement of stimulating and recording electrodes. Extracellular potentials were recorded by 3.0 M KCl-filled micropipettes (2-4 MΩ; 1 µm inside diameter). Potentials were amplified with an Axon Instruments Multiclamp 700A amplifier (Union City, CA). Microelectrodes were oriented, via stereotaxic coordinates, into the VTA (from bregma: 5.6 - 6.5 posterior (P), 0.5 - 1.0 lateral (L), 6.5 - 7.5 ventral (V)) with a piezoelectric microdrive (EXFO Burleigh 8200 controller and Inchworm, Victor, NY).

Single-Cell Electrophysiology

Single-unit activity was filtered at 0.3 - 10 kHz (-3dB) and amplified with the Multiclamp 700A amplifier and displayed on Tektronix digital oscilloscopes (Beaverton, OR). Potentials were sampled at 20 kHz (12 bit resolution) with National Instruments Data Acquisition Boards in Macintosh computers (Apple Computer, Cupertino, CA). Extracellularly-recorded action potentials were discriminated with a World Precision Instruments, WP-121 Spike Discrimator (Sarasota, Fl) and converted to computer-level pulses. Single-unit potentials, discriminated spikes, and stimulation events were captured by National Instruments NB-MIO-16 digital I/O and Counter/Timer Data Acquisition Boards (Austin, TX) in Macintosh computers.

Characterization of VTA GABA Neurons In Vivo

VTA GABA neurons were identified by previously-established stereotaxic coordinates, by spontaneous and electrophysiological criteria (S. C. Steffensen, et al., 1998), in response to *in situ* iontophoretic dopamine (S.C. Steffensen, et al., 2003). The neurons are distinguished by stimulation of their principal excitatory afferent and discharge profile. They included: relatively fast firing rate (>10Hz); ON-OFF phasic non-bursting activity; spike duration less than 200 µsec; and multiple post-stimulus spike discharges (PSDs) produced by stimulation of the internal

capsule(IC; coordinates: -1.0-1.3 P, 2.3-3.0 L, 5.0-6.0 V) (ICPSDs). Activation of the IC was accomplished by stimulating with insulated, bipolar stainless-steel electrodes in which square-wave constant current stimulus pulses are provided (500-1000 μA; 0.15 ms duration; average frequency, 0.1 Hz) that was generated by an AMPI IsoFlex isolation unit controlled by an AMPI MASTER-8 Pulse Generator (Jerusalem, Israel). We evaluated only those spikes that had greater than 5:1 signal-to-noise ratio and were driven by IC stimulation. Stimulation was performed at an intensity that produces 50% maximum VTA GABA neuron ICPSDs with 10 pulses at 200 Hz (S.C. Steffensen, et al., 2003; S. C. Steffensen, et al., 1998).

Acupuncture Stimulation

Stainless-steel needles with a diameter of 0.18 mm and a length of 50mm were inserted vertically to a depth of 3 mm into acupuncture points HT7, PC6 and Tail. The anatomical location of acupuncture points stimulated in rats corresponded to the acupoints in man as described previously (Stux, Berman, & Pomeranz, 2003) and in animal acupuncture atlas (Schoen, 2001). HT7 is anatomically located on the transverse crease of the wrist of the forepaw, radial to the tendon of the m. flexor carpi ulnaris. PC6 is located between the tendons of the m. palmaris longus and flexor carpi radalis, 4 mm proximal to the transverse crease of the wrist of the forepaw. For single-unit studies, acupuncture was accomplished with finely-controlled electrical stimulation. Insulated tungsten sharp microelectrodes (1-5 MΩ; ET-10-5, A-M Systems, Everette, WA) were inserted orthogonally into HT7 around 3 mm from skin surface. For Tail stimulation, electrodes were inserted bilaterally at the base of the Tail at a 30 degree angle to a depth of 2-3 mm. Activation of acupuncture points and non-acupuncture points was accomplished by stimulating with square-wave constant current stimulus pulses (1.0 msec duration; frequency, 2 Hz) that were generated by an AMPI IsoFlex isolation unit that were

controlled by an AMPI MASTER-8 Pulse Generator. The stimulation current was adjusted until muscle twitch was visually detected (300-800 μA) either at the digits for HT7 stimulation or at the Tail muscles for Tail stimulation. For acupuncture treatment in freely-behaving rats, acupuncture was accomplished with mechanical stimulation. Researchers have used the same acupuncture paradigm as described by a previous study (R. J. Zhao, et al., 2006). Stainless-steel needles (0.18 mm diameter and 20 mm length) were inserted vertically to a depth of 3 mm into acupuncture points of rats lightly restrained by hands for 1-min under unanesthetized condition. Since the previous work showed that a brief manual acupuncture (1 min) was effective in reducing ethanol-induced DA release in the NAcc (Yoon, et al., 2004), and both electroacupuncture and manual acupuncture may recruit the identical peripheral afferent mechanisms involved in acupuncture analgesia (Z. Q. Zhao, 2008), rats were subjected to manual acupuncture at bilateral HT7 for 1 min. The acupuncture stimulation was manually delivered by twisting acupuncture needles at a frequency of twice per sec for a total of two sec of stimulation while needles were inserted and withdrawn from acupoints. Also, acupuncture was applied at non-acupoints one-fifth of Tail length from the proximal region of the Tail to avoid the two Tail acupoints. In one group of rats, the Tail was used as a stimulation control site to determine the effect of mechanical stimulation at non-acupoints. In another group of rats, Neiguan (PC6) was used as nonspecific control points. In the control group, rats were lightly restrained by hands in the same method as that used for each acupuncture treatment group but acupuncture was not applied. The rats were pre-handled for 2 min/day for 5 consecutive days before exposure to acupuncture for the reduction of stress.

Dopamine Voltammetric Experiments

For single-unit studies, systemic administration of 1.0 g/kg ethanol (16% w/v ethanol in saline) was accomplished by intraperitoneal injections. Non-selective OR antagonists, naloxone hydrochloride and naltrindole hydrochloride were dissolved in saline at 1.0 mg/mL and 15 mg/mL, respectively, and administered intravenously at a volume corresponding to each rat's weight in μL/gm. An equal volume of saline was administered intravenously to a paired rat for comparisons of ethanol effects on VTA GABA neuron firing rate.

Dopamine Voltammetric Experiments

Fast-scan cyclic voltammetry (FSCV) is an electrochemical method for measuring concentrations of cyclic compounds such as DA, serotonin, and epinephrine. By measuring the concentrations of these endogenous neurotransmitters in the brain areas associated with drug addiction, we expect to find the cellular mechanism that lead to addiction. Electrically active compounds are oxidized and reduced at specific values. The oxidation or reduction of the chemical compounds can then be measured by the respective change in current. The amplitude of the current is representative of the concentration of the chemical. In our experiment, we used FSCV to measure the DA. Because concentrations of a neurotransmitter are often dependent on whether a pathway is being activated, DA FSCV can be used to find physiological markers for the neurological mechanisms such as reward and addiction. Carbon fiber electrodes (CFEs) were constructed by pulling carbon fiber in a glass pipette blank on a Narishige puller. The tip of the pulled CFE was cut to 100 microns under microscopic control with a razor blade.

Voltammetric recordings were made at the CFE every 100 msec by applying a triangular waveform (0.4 to +1.3 V, 300-400 V/s) using a biopotentiostat (ChemClamp; Dagan Corporation, Minneapolis, MN). Data were digitized (National Instruments, Austin, TX) and

stored to a computer. Dopamine release was evoked every 5 min with electrical stimulations (60 rectangular biphasic pulses, 60 Hz, 120-500 μA, 2 msec/phase) and detected at the CFE. After at least three stimulations, a single dose of ethanol was injected. Stimulations and recordings continued at 10-min intervals for 60 min postinjection. The CFEs were calibrated with a known dose of dopamine (3 uM) *in vitro* after each experiment. To pharmacologically confirm that the signal detected is DA, some rats received GBR 12909, a potent and selective DRI that blocks DA uptake, after ethanol administration. DA uptake follows Michaelis-Menten kinetics and is thus concentration-dependent. Thus, comparison of uptake following evoked release requires analysis of clearance rates at the same absolute DA concentration. To determine qualitatively the effect of ethanol on DA uptake, three pre-ethanol evoked responses were averaged, truncated to the range of concentrations observed after ethanol, and compared with the post-ethanol responses. To obtain a more quantitative measure of uptake, the slope of the descending phase of the evoked DA signal was used. The slope was measured as the tangent taken from six points (500 msec). For data from each animal, three slopes obtained before ethanol were averaged and compared with the average slopes determined 40, 50, and 60 min after ethanol. These slopes were compared with a paired *t* test at each dose.

Chronic Ethanol Self-Administration

To investigate the effects of HT7 acupuncture on chronic ethanol consumption over DA release in the NAcc, a specific dietary method, known as the Lieber-DeCarli diet (Lieber & DeCarli, 1989), was used. This method of feeding ethanol has been evaluated quite effective in that ethanol intake is consistently increased. In addition, it has also shown that physical dependence and withdrawl may be followed as a consequence. When systemically administered in a condition in which other spontaneous food and drink are not provided, ethanol intake may

reach up to 18 g/kg per day (Lieber & DeCarli, 1989). In this experiment, it involved making a diet with ethanol, measuring the consumption each day in the ethanol rats, and adjusting the amount of the diet given to control pair-fed rats. In the experiment, 6 rats were used for either ethanol or control group for 3 weeks and the DA levels were measured for each pair of rats on the altar following the diet. Once weighing over 200 g (34-38 days old), rats were housed separately in different cages and given *ad libitum* access to solid food and water (Dyets, Bethlehem PA). After one day, the solid diet and water were removed from cages and rats were randomly assigned to either control pair-fed or chronic ethanol group. The liquid diet, composed of maltose dextrin, Lieber DeCarli powder, and water, was made up fresh every few days in a blender and stored at 4ºC in a refrigerator. The amounts of the diet consumed from each group were recorded in a 24-hr cycle. The control group received the standard liquid diet and the chronic ethanol group received an ethanol-containing diet. Due to the natural aversion of rats to ethanol, the rats in the ethanol group were introduced to the ethanol by increasing the percentage of ethanol incrementally, from 3% to 5%, over a 5 day period. To accommodate for reduced food intake, the chronic ethanol rats were pair-fed with the control group to maintain equal caloric intakes in both groups, with the control group only receiving the amount their pair-fed chronic ethanol rats consumed on the previous day. In addition, maltose dextrin was added to the control diet to make the diet iso-caloric with the ethanol diet. Once the chronic ethanol group reaches an ethanol level of 5% in their liquid diet, they were maintained at this concentration for two weeks. Both control and ethanol liquid diets were changed daily at 5:00 p.m. Prior to *in vivo* electrophysiology experiments, chronic ethanol rats were withdrawn from ethanol for a period of 8 hours. Each day, a pair of rats from in both ethanol diet and pair-fed control group were withdrawn for measurement DA release in the NAcc (from bregma: 1.7 P, 1.0 L, 6.5 V) by

FSCV, while those remained in the cage were substituted with water for the diet. In order to evaluate the effects of ethanol consumption on DA signals, the rats were set up for MFB-NAcc shell DA FSCV. After a signal was obtained, we tested the effects of HT7 stimulation on the relationship between chronic ethanol consumption and DA neurons in NAcc. Because nothing seems to have occurred to ethanol- naïve rats in the past, we tried to assess the effects of HT7 and ethanol administered chronically in the diet in ethanol- treated rats compared with their pair-fed controls. We challenged the rats with a dose of ethanol, 2 g/kg, as it significantly decreases DA FSCV in ethanol untreated rats.

Chronic Ethanol Injection

Empirically, chronic ethanol injection is believed to induce a state of dependence in the VTA mediating the rewarding properties of ethanol. As a recent study (Yang, et al., 2008) reported that acupuncture at a specific point blocks DA depletion, as assessed by microdialysis, during withdrawal from chronic ethanol, we evaluated the effects of HT7 stimulation on DA depletion by voltammetry. Among those weighing over 300g, six rats were chosen. They were divided into two groups, either ethanol or saline in an *ad libitum* access to solid food and water in the cage. In the experiment, with instantaneous anesthetization, rats were injected intraperitoneally twice daily (08:00 hrs, 17:00 hrs) with 3.0 g/kg ethanol or saline for three weeks. Eight hours following withdrawal from ethanol or saline injection, rats were anesthetized on the altar, and both release and uptake in DA levels were evaluated by voltammetry in the shell region of the NAcc with MFB stimulation (from bregma: -1.3 P, 2.0 L, 7.8 V). We first evaluated baseline DA levels of ethanol injection and compared to chronic saline injection. Then, we monitored the effects of HT7 stimulation on VTA GABA neuron firing rate, which we expected would be elevated, due to adaptation of VTA GABA neurons with chronic ethanol

(Gallegos et al, 1999). We challenged each withdrawing rat with 1.0 g/kg ethanol during continuous HT7 stimulation to see if HT7 block of ethanol inhibition of VTA GABA neuron activity has adapted with chronic ethanol.

Analysis of Responses and Statistics

For analysis of single-unit data, discriminated spikes and stimulation events were processed with National Instruments LabVIEW and IGOR Pro software (Wavemetrics, Lake Oswego, OR). Extracellularly recorded single-unit action potentials were discriminated by a peak detector digital processing LabVIEW algorithm. The effects of HT7 stimulation on VTA GABA neuron activity were determined by rectangular activation of ratemeter records over 5 min of stimulation with IGOR Pro software. To determine changes in VTA GABA neuron firing rate produced by ethanol administration, firing rate was determined by averaging 5 min epochs of activity before and 5 min epochs at 10 min after ethanol injection, by rectangular integration of ratemeter records with IGOR Pro software. The results for control and drug treatment groups were derived from calculations performed on ratemeter records and expressed as means ± S.E.M. A paired two sample for means t test was performed to determine statistical significance for within-subject drug versus saline comparisons with Microsoft Excel Statistical Analysis Toolpak and IGOR Pro (Wavemetrics, Oswego, OR) Stat Pak with $\alpha = .05$ and in 95% CI. A simple one-way ANOVA was used to compare the effects of drugs with that of saline for between-subjects firing rate. Figures were compiled by using IGOR Pro Software. For analysis of behavioral testing, statistical analysis of data was performed using SPSS. One-way ANOVA and post-hoc Tukey tests were carried out to compare the control group and each acupuncture treatment group. For comparison of sucrose self-administration, a paired *t* test was performed. Baseline

responding was calculated as the mean absolute ethanol-paired lever responding from three consecutive responses exhibiting less than 20% difference.

Results

Effects of HT7 Stimulation on VTA GABA Neuron Firing Rate

We compared the effects of HT7 and Tail electrical stimulation on the firing rate of VTA GABA neurons, which were characterized by previously-established criteria including spontaneous activity, waveform properties, and response to internal capsule (IC) stimulation (S. C. Steffensen, et al., 1998). Both Tail and HT7 electrical stimulation (2 Hz at threshold current for muscle twitch) produced transient increases in VTA GABA neuron firing rate, typically at the onset of stimulation (see Figure 3A,B). Of the 55 neurons tested for effects of HT7 stimulation on VTA GABA neuron activity, 24 (44%) showed an initial (EARLY) increase in firing rate (47.3 ± 9.6%) followed by a more sustained (LATE) decrease (52.5 ± 7.3%) in firing rate with recovery in 5 min (see Figure 3C**;** baseline firing rate for HT7 stimulation was 34.9 ± 2.5 Hz). Of the 15 neurons tested for effects of Tail stimulation on VTA GABA neuron activity, 14 neurons (93%) showed an EARLY increase (64.2 ± 23%) in firing rate, similar to HT7 stimulation. However, none showed a LATE inhibition (0.7 ± 4.2%) that was characteristic of HT7 stimulation (see Figure 3C**;** baseline firing rate for Tail stimulation was 35.9 ± 4.2 Hz). Although there was no significant difference in EARLY excitation between HT7 and Tail stimulation ($p > .05$), there was a significant difference in LATE inhibition between HT7 and Tail stimulation ($p = 7.1E\text{-}06$, $F(1,30) = 29.80$)**.**

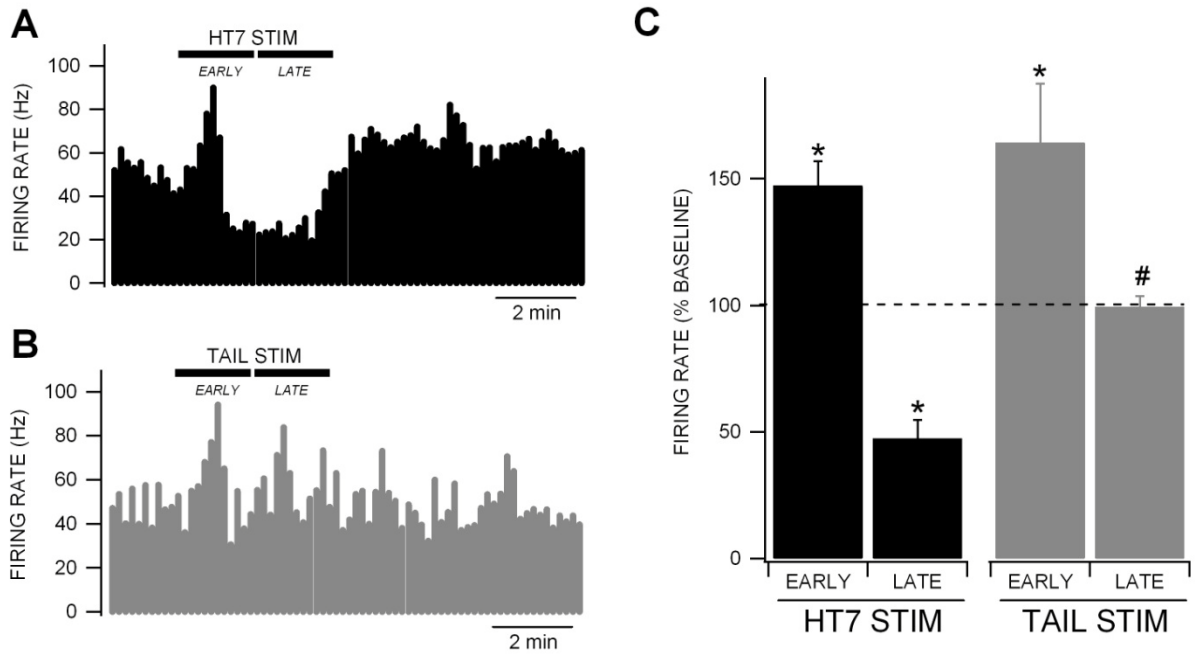


Figure 3. **Modulation of VTA GABA neuron firing rate by HT7 vs. Tail electrical stimulation**. (A) This ratemeter record shows a representative VTA GABA neuron with a baseline firing rate of approximately 50 Hz. HT7 electrical (2 Hz at threshold current for muscle twitch) stimulation produced an initial (EARLY) enhancement of VTA GABA neuron firing rate followed by a more prolonged (LATE) inhibition, with subsequent recovery in 5 min. (B) This ratemeter record shows the same neuron during Tail stimulation. Tail electrical stimulation produces only an EARLY increase in firing rate. (C) Summary of HT7 and Tail stimulation on VTA GABA neuron firing rate. HT7 stimulation produced a significant EARLY excitation (n = 24) followed by a significant LATE inhibition of VTA GABA neuron firing rate (n = 18). Tail stimulation produced only a significant EARLY excitation (n = 14). There was a significant difference between HT7 and Tail LATE effects on VTA GABA neuron firing rate. Asterisks * represents $p < .05$ compared to baseline and # represents p = 7.2E-06 between HT7 vs. Tail LATE responses.

Effects of Naloxone on HT7 Inhibition of VTA GABA Neuron Firing Rate

Because HT7 stimulation inhibited VTA GABA neuron firing rate similar to what we have demonstrated previously with opiates (S. C. Steffensen, et al., 2006), we tested the effects of the non-selective MOR antagonist, naloxone on HT7 modulation of VTA GABA neuron firing rate. Compared to saline, intravenous administration of 1.0 mg/kg naloxone slightly, but not significantly, increased VTA GABA neuron firing rate (14.5 ± 10.2%; p = .50; n = 18). Naloxone blocked the LATE inhibition produced by HT7 stimulation, but did not affect the EARLY enhancement of VTA GABA neuron firing rate (see Figure 4A). Intravenous administration of saline (isovolumic with naloxone injection) had no effect on ability of HT7 modulation to reduce VTA GABA neuron firing rate (see Figure 4B). There was a significant difference between naloxone and saline on the LATE inhibition of VTA GABA neuron firing rate produced by HT7 stimulation (p = .006, $F(1,11)$ = 12.1; n = 6,7, respectively).

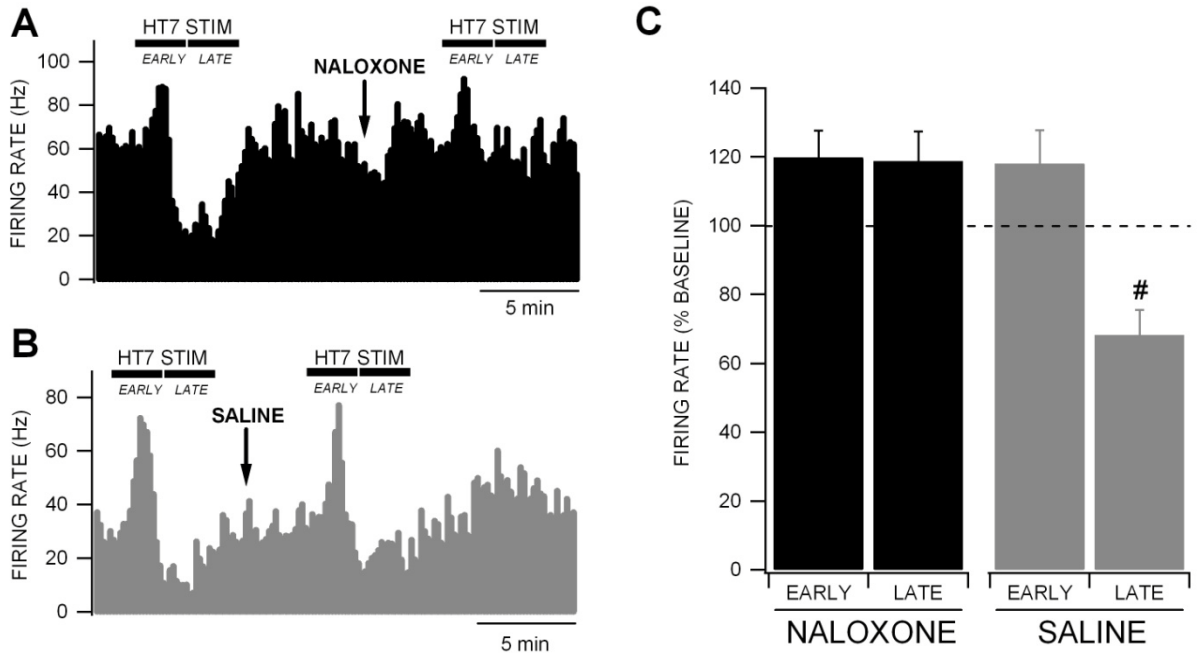


***Figure 4*. Naloxone blocks the inhibition of VTA GABA neuron firing rate by HT7 electrical stimulation**. (A) This ratemeter record shows a representative VTA GABA neuron with a baseline firing rate of approximately 65 Hz. Intravenous administration of the MOR antagonist naloxone blocked the LATE inhibition, but not the EARLY activation produced by HT7 electrical stimulation. (B) This ratemeter record shows a VTA GABA neuron in a separate experiment with a baseline firing rate of approximately 30 Hz. Intravenous administration of saline (isovolumic to naloxone injection) did not affect either EARLY or LATE phases of HT7 stimulation. (C) There was a significant difference between saline (n = 7) and naloxone (n = 6) on the LATE inhibition of VTA GABA neuron firing rate. Most important, there was a significant difference between naloxone and saline in the inhibition of HT7 stimulation LATE effects on VTA GABA neuron firing rate.

Effects of HT7 Stimulation on Ethanol Inhibition of VTA GABA Neuron Firing Rate: Role for MOR

We then examined the effects of continuous HT7 and Tail stimulation on ethanol inhibition of firing rate of VTA GABA neurons. We and other researchers have previously reported that acute intoxicating doses of ethanol inhibit VTA GABA neuron firing rate with an IC_{50} of 1.0 g/kg (Gallegos, et al., 1999; Ludlow, et al., 2009; S. C. Steffensen, et al., 2009; Stobbs, et al., 2004). Thus, we evaluated the effects of HT7 stimulation at the IC_{50} dose for ethanol. Ten min after HT7 or Tail stimulation, a dose of 1.0 g/kg ethanol was administered intraperitoneally. The GABA neurons that were inhibited by HT7 stimulation were studied. Only one neuron was studied in each rat and HT7 or Tail stimulation was administered continuously throughout each experiment. The figure below shows the effects of continuous HT7 or Tail (Figure 5A, 5B) stimulation on acute ethanol inhibition of VTA GABA neuron firing rate. While this dose of ethanol markedly decreased VTA GABA neuron firing rate in rats receiving Tail stimulation (61.8 ± 13.6%), there was an increase in firing rate in those receiving

HT7 stimulation (48.8 ± 25.6%; Figure 5C). There was a significant difference in ethanol effects between HT7-stimulated and Tail-stimulated rats (Figure 5C; p = .002, $F(1,14) = 15.7$; n = 7,8, respectively). Since naloxone abolished the inhibition produced by HT7 stimulation, we evaluated its effects on HT7 block of ethanol inhibition of VTA GABA neuron firing rate. Compared to saline, intravenous administration of naloxone (1.0 mg/kg) restored ethanol's ability to suppress the firing rate of VTA GABA neurons (Figure 5D; saline with naloxone: p = .001, $F(1,17) = 17.1$; n = 11,7, respectively).

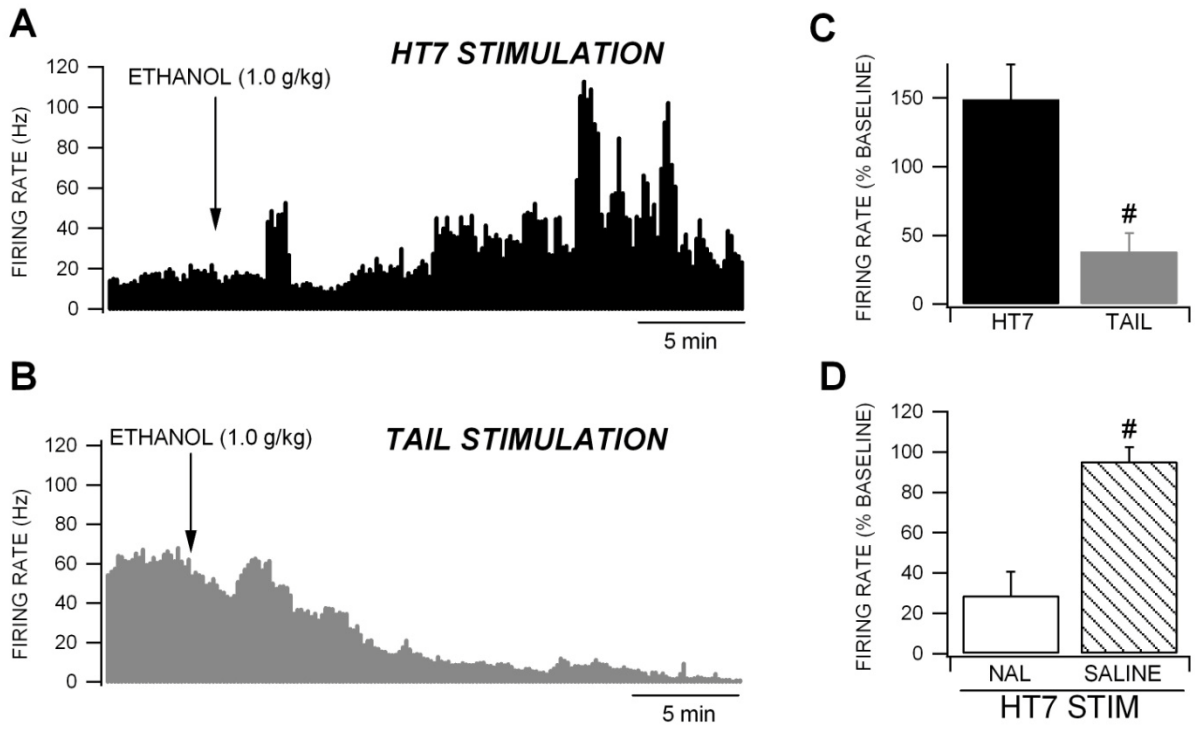


***Figure 5*. Comparison between continuous HT7 and Tail electrical stimulation on acute ethanol inhibition of VTA GABA neuron firing rate.** (A) This ratemeter record shows a representative VTA

GABA neuron with a baseline firing rate of approximately 19 Hz during continuous HT7 electrical stimulation. Intraperitoneal administration of 1.0 g/kg ethanol produced a prolonged enhancement of this neuron's activity during HT7 stimulation. (B) This ratemeter shows the firing rate of a VTA GABA neuron in a separate experiment with a baseline firing rate of 60 Hz during continuous tail stimulation. Intraperitoneal administration of 1.0 g/kg ethanol produced its typical inhibition of firing rate (Gallegos, et al., 1999; Ludlow, et al., 2009; S. C. Steffensen, et al., 2009; Stobbs, et al., 2004). (C) This graph summarizes the effects of HT7 vs. Tail stimulation on ethanol inhibition of VTA GABA neuron firing rate. There was an increase in firing rate produced by ethanol during continuous HT7 stimulation (n = 7). Ethanol produced its typical inhibition during continuous Tail stimulation (n = 8). There was a significant difference in ethanol effects between HT7 vs. Tail electric stimulation (p = .002). (D) There was a significant difference between naloxone vs. saline on HT7 block of ethanol inhibition of VTA GABA neuron firing rate (p = .001). # represents p = .0008 between naloxone vs. saline effects.

Role for Delta-Opioid Receptors in Ethanol Inhibition of VTA GABA Neuron Activity

We compared the effect of non-selective MOR antagonist, naloxone and that of the DOR antagonist, naltrindole on both VTA GABA neuron firing rate and ethanol inhibition of their firing rate. Compared to saline, intravenous administration of 15 mg/kg naltrindole slightly, but not significantly, decreased VTA GABA neuron firing rate (11.5 ± 13.1%; p = .5; n = 13). Also, there was no statistically significant difference between naloxone and naltrindole in baseline firing rate (naloxone with naltrindole: p = .12, $F(1,30) = 2.5$; n = 13,18, respectively). Naloxone administered 15 min prior to an intraperitoneal injection of 1.0 g/kg ethanol slightly, but not significantly, enhanced ethanol's inhibition of VTA GABA neuron firing rate (see Figure 6A), Naltrindole (15 mg/kg) administered 15 min prior to an intraperitoneal injection of 1.0 g/kg ethanol abolished ethanol's inhibition of VTA GABA neuron firing rate (see Figure 6B). There was a significant difference between naloxone and naltrindole for ethanol effects on VTA GABA

neuron firing rate (see Figure 6C; naloxone with naltrindole: p = .001, $F(1,26) = 13.7$; n = 12, 15, respectively).

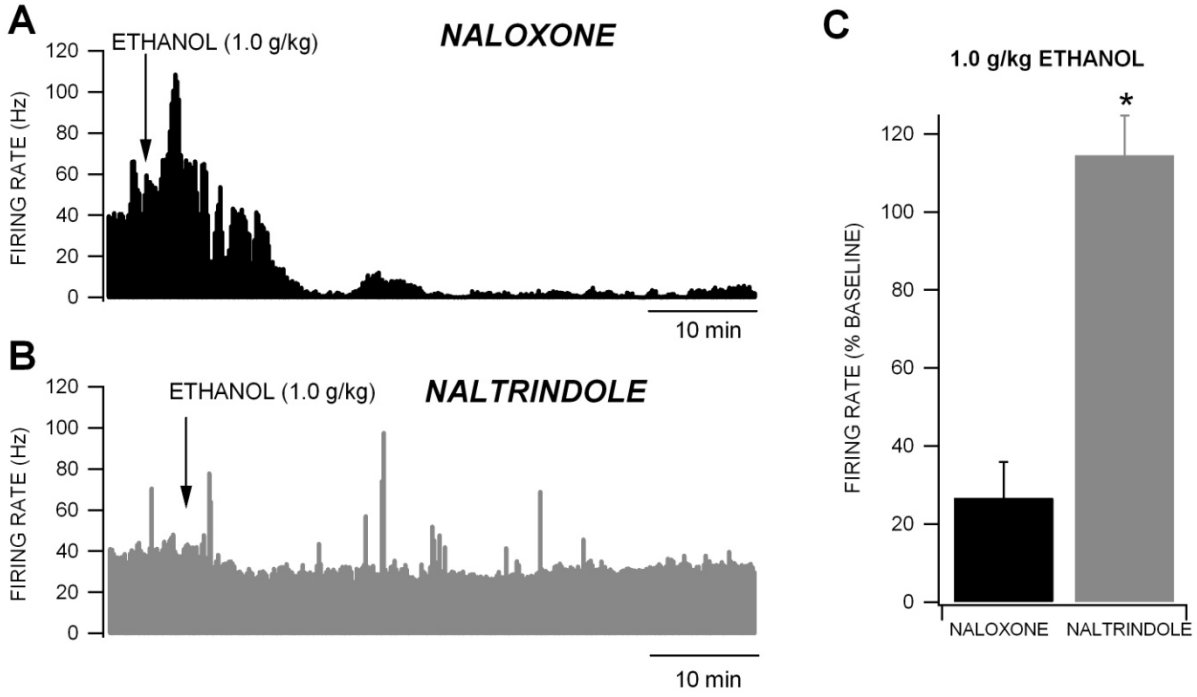


Figure 6. **Naltrindole, but not naloxone, blocks ethanol inhibition of VTA GABA neuron firing rate.** (A) This ratemeter record shows a representative VTA GABA neuron with a baseline firing rate of approximately 37 Hz in the presence of 1.0 g/kg naloxone. Intraperitoneal administration of 1.0 g/kg ethanol produced its typical inhibition of VTA GABA neuron firing rate when naloxone is present. (B) This ratemeter shows the firing rate of a VTA GABA neuron in a separate experiment with a baseline firing rate of 39 Hz in the presence of 15 mg/kg naltrindole, which blocked the inhibition of VTA GABA neuron firing rate produced by 1.0 g/kg ethanol. (C) This graph summarizes the effects of naloxone and naltrindole on ethanol inhibition of VTA GABA neuron firing rate. Naltrindole significantly reduced ethanol inhibition of VTA GABA neuron firing rate. * represents p = .001 between naloxone vs. naltrindole effects.

Effects of Acupuncture at HT7 on Ethanol-Reinforced Responding

Our collaborators in Korea evaluated the effects of HT7, Tail, and PC6 acupuncture on ethanol-reinforced responding. The ethanol-reinforced responding and the amount of ethanol consumption across the last 3 sessions before acupuncture did not vary significantly by group ($p > .05$; control: 27.1 ± 3.0 responses, 0.44 ± 0.05 g/kg, $n = 8$; HT7: 29.1 ± 2.9 responses, 0.47 ± 0.05 g/kg, $n = 8$; Tail: 23.8 ± 1.9 responses, 0.50 ± 0.05 g/kg, $n = 9$; and PC6: 30.6 ± 3.1 responses, 0.42 ± 0.03 g/kg; $n = 7$). However, one-way ANOVA analysis revealed a significant main effect of acupuncture treatment ($F(3,28) = 10.55$, $p < .001$). Post hoc Tukey tests showed that there was a significant reduction in the amount of ethanol consumption in the HT7 group compared to the control group ($p < .01$; control = 0.50 ± 0.05 g/kg with HT7 = 0.23 ± 0.02 g/kg; see Figure 7A). This effect of HT7 did not seem to be a long-term effect as the baseline responding for ethanol returned on the following day. Additionally, Post hoc Tukey tests also showed that HT7 acupuncture produced a significant decrease in ethanol-reinforced responding compared to acupuncture at PC6 ($p < .05$; PC6 = 0.45 ± 0.06 g/kg; Figure 7A). The HT7 group demonstrated initial suppression of ethanol responding and highly significant decreases in cumulative responses starting at 5 min and continuing to the end of the test session at 30 min compared with the control group (see Figure 7B; $n = 8$). To control for the possibility that acupuncture causes generalized suppression of operant responding, sucrose self-administration was performed using ethanol-naive rats. Before HT7 acupuncture treatment, the baseline values for each treatment group were 20.9 ± 1.13 min for control and 19.5 ± 0.71 min for HT7, respectively. Acupuncture at HT7 did not alter the response rate of sucrose self-administration compared to control group (Figure 7C; t_5=.426, $p = .208$; $n = 6$). These results suggest that

reduction in ethanol-reinforced responding by HT7 acupuncture cannot be attributed to motor impairment.

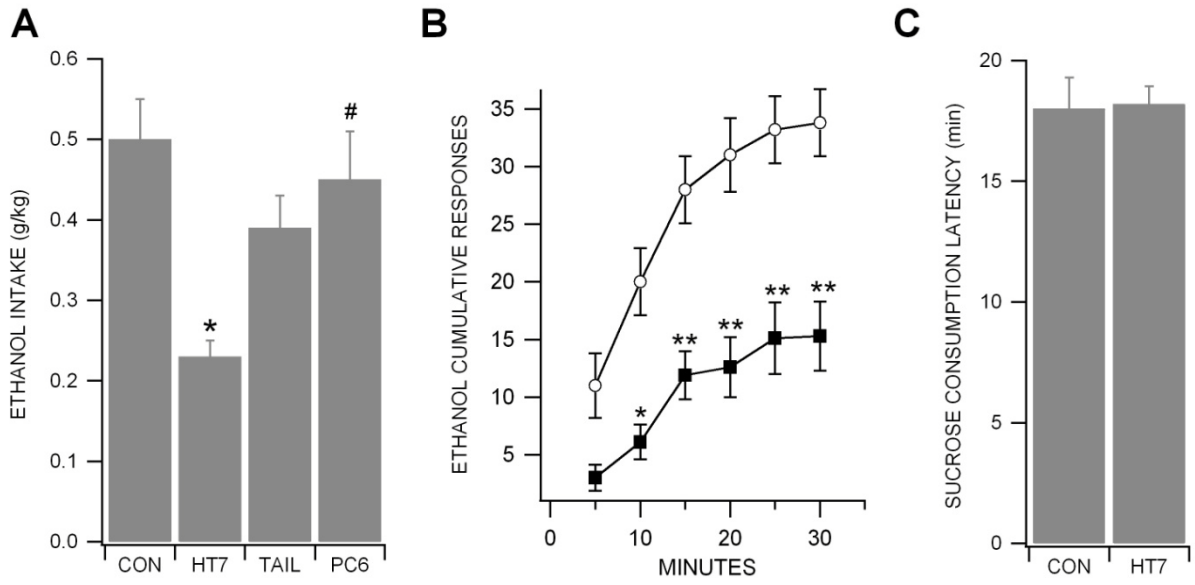


Figure 7 **Acupuncture at HT7 reduces ethanol, but not sucrose, reinforced behavior**. (A) Rats manually received the stimulation of bilateral HT7 (Shenmen, *n* = 8) points for 1 min immediately before testing session. Acupuncture points corresponding to bilateral Tail (Tail, *n* = 9) and PC6 (Neiguan, *n* = 7) points were used as control points. Results are expressed as the mean ± S.E.M. for the amount of ethanol consumption (in g/kg) during the 30 min self-administration session. HT7, but not Tail or PC6, stimulation significantly reduced ethanol intake (**p* < .01, control group vs. HT7 group; #*p* < .05, HT7 group vs. PC6 group. (B) Cumulative responses for ethanol during the entire 30 min session time after HT7 stimulation (*n* = 8). HT7 acupuncture significantly reduces ethanol self-administration during the entire session time, except 5 min after starting of the session compared to control (*n* = 8). Asterisks *, ** represent *p* < .01 and *p* < .001 between control group vs. HT7 group, respectively. (C) To control for the possibility that acupuncture affects generalized suppression of operant responding, sucrose pellet self-administration was performed using ethanol-naive rats (*n* = 6). HT7 acupuncture did not alter the time to self-administer 50 sucrose pellets.

Effects of Naltrindole on Ethanol-Reinforced Responding

To determine the effects of the DOR antagonist, naltrindole on ethanol consumption, rats were given saline or naltrindole (15 mg/kg) 30 min before the start of ethanol self-administration. There was no significant difference in baseline ethanol consumption between groups (p = .213; saline group: 0.61 ± 0.07 g/kg, n = 6; naltrindole group; 0.54 ± 0.08 g/kg, n = 6). Intraperitoneal administration of naltrindole markedly reduced ethanol self-administration (t_5 = 4.163, p < .01; n = 6) without affecting the latency to self-administer 50 sucrose pellets compared to saline group (t_4 = .1367, p = .8978; n = 5), suggesting that naltrindole did not produce generalized effects on response rates.

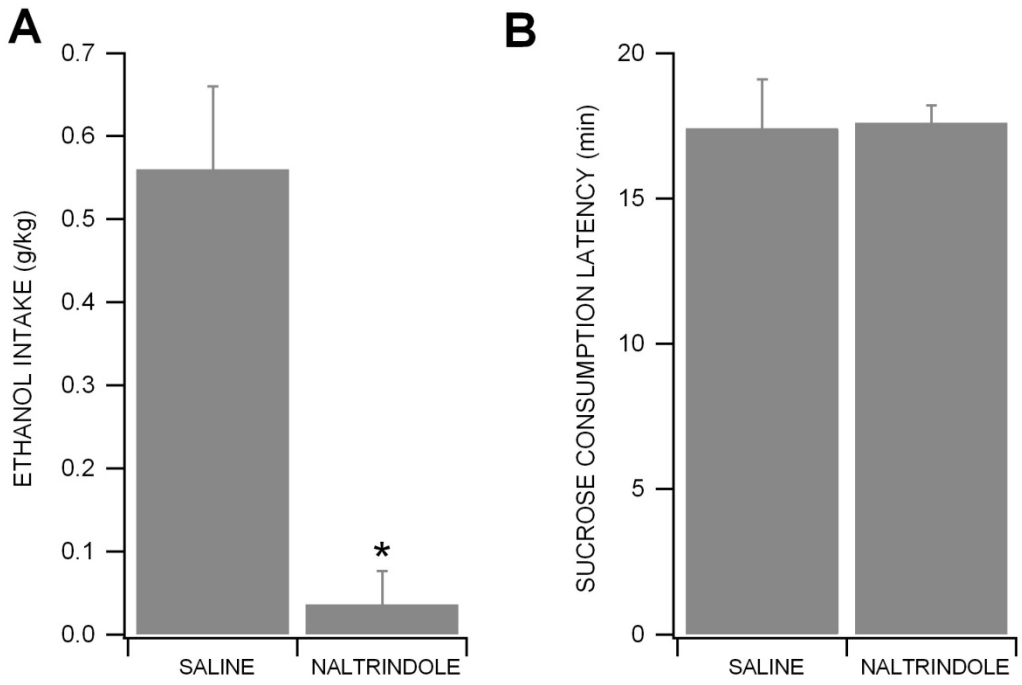


Figure 8 **Naltrindole reduces ethanol self-administration**. (A) This figure shows the effects of naltrindole on ethanol self-administration. Intraperitoneal injection of naltrindole (n = 6) but not saline (n = 6) significantly suppressed ethanol-reinforced responding. Data represent the mean ± S.E.M. of ethanol consumption. * represents p = .01 between saline vs. naltrindole effects (paired t test). (B) This graph

shows the latency to self-administer 50 sucrose pellets. Data represent the mean ± S.E.M. of the time to consume 50 sucrose pellets. Neither saline (n = 5) nor naltrindole (n = 5) did alter response rates of sucrose self-administration.

Effects of Chronic Ethanol in both Independent and Dependent Condition on DA Release in the NAcc

Even though verified in the previous studies using the diet, it was important to see if it would be a feasible treatment strategy that doesn't have a negative effect on the overall health of the rats before electrophysiological procedure including FSCV measurements of DA release and uptake in the NAcc in the presence of ethanol administration or electroacupuncture stimulation. We found that ethanol-treated rats had body weights that did not differ significantly from those of their pair-fed controls (p = .44, $F(1,5)$=1.27, n = 6), suggesting that the chronic ethanol treatment did not adversely affect the dietary intake of calories (see Figure 9). This is consistent with previous studies using the Lieber-DeCarli diet and the average daily growth rate for both groups was approximately 3 grams per day. The average consumption of ethanol during the 3 week period for the ethanol diet rats was 9.94 g/kg/day while that of diet without ethanol for pair-fed rats was slightly high, 10.58 g/kg daily.

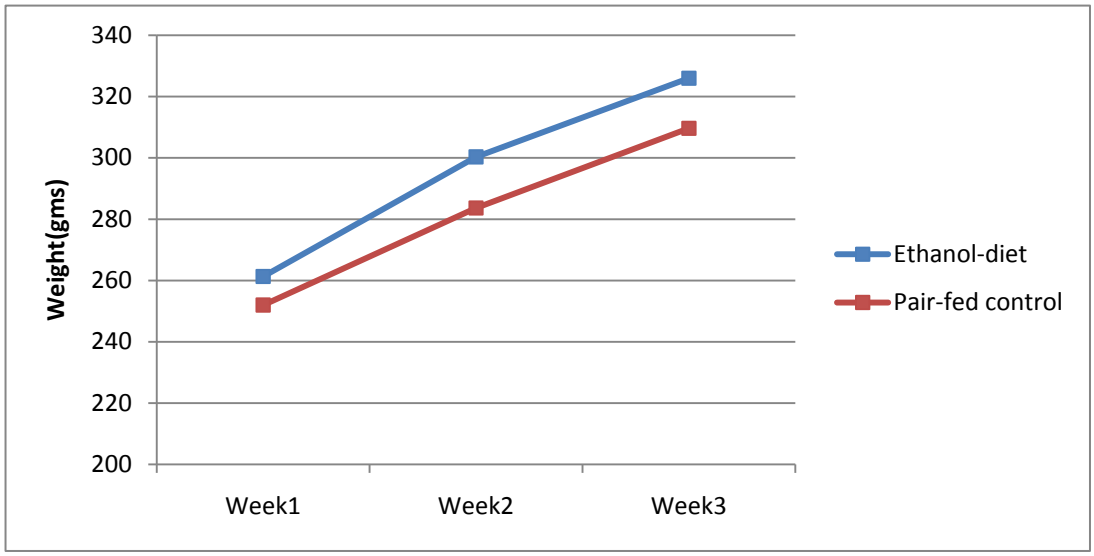


Figure 9. **Comparison of average weight- gain**. There was no significant difference in weight-gain between chronic ethanol treated rats and pair-fed control rats, using Lieber DeCarli Diet. As suggested in a previous study (Ludlow et. al, 2009), administration of chronic ethanol in the diet didn't affect adversely the overall health of animals.

Only four out of six rats in pairs were used to measure the release DA neurons in the NAcc for each treatment group after being withdrawn from the dietary intake. But, even with electrical stimulation at HT7 acupuncture following MFB activation, there was no significant difference in the level of DA neurons between chronic ethanol diet rats and pair-fed rats; either decrease or maintain the amounts of release of DA neurons. And despite the subsequent challenge of acute administration of ethanol in the presence of continuous HT7 acupuncture stimulation, we couldn't find any consistent and comparable data of DA release or depletion for both ethanol treated rats and their pair-fed. Nonetheless, there was a significant difference in the variance of DA levels in the pairs between two groups ($p < .001$; $n = 4$). We found it interesting that chronic ethanol-containing diet rats showed remarkably the higher DA neurons levels than those administered with the ethanol- free diet (see Table 2). And then, we set up a separate

experiment with a different condition, in a dependent state to assess the effect of HT7 stimulation on DA depletion in the NAcc. Either 4. 38 ml of ethanol or isovolumic amount of saline was injected to the rats daily immediately after 1 min anesthetization with isoflorine. Unfortunately, continuous intraperitoneal injection of ethanol resulted in the sickness or death of rats in ethanol injection group. Thus, we had only 4 rats in pairs for electrophysiological procedure to record the change of DA neurons in the region. Unlike from those in independent condition in which ethanol was administered in a diet, the level of DA neurons in chronic ethanol injection rats was significantly lower than that obtained in chronic saline injection rats, regardless of an acute ethanol challenge following HT7 acupuncture and MFB stimulations. But, as indicated in the figure below **(**see Figure 10**)**, there was no significant difference in the increase of DA neurons between chronic ethanol injection rats and those rats with chronic saline (p = .0621; n = 4).

Table 2

The DA levels in the NAcc in independent condition, using Lieber DeCarli Diet

	HT7 stimulation		Acute ethanol	
	0 min.	30 min.	0 min.	10 min.
Pair-fed (1)	63.63	58.09	68.86	69.34
Ethanol (1)	123.64	106.40	140.65	129.15
Pair-fed (2)	19.12	19.49	12.20	27.02
Ethanol (2)	42.90	42.97	39.66	48.24

Note. The DA release with MFB stimulation followed by HT7stimulation and a subsequent acute ethanol administration was measured by voltammetry. The concentration of DA signals was evaluated in currents (nA). The numbers in parentheses are the order of the rats used for the experiment in each group.

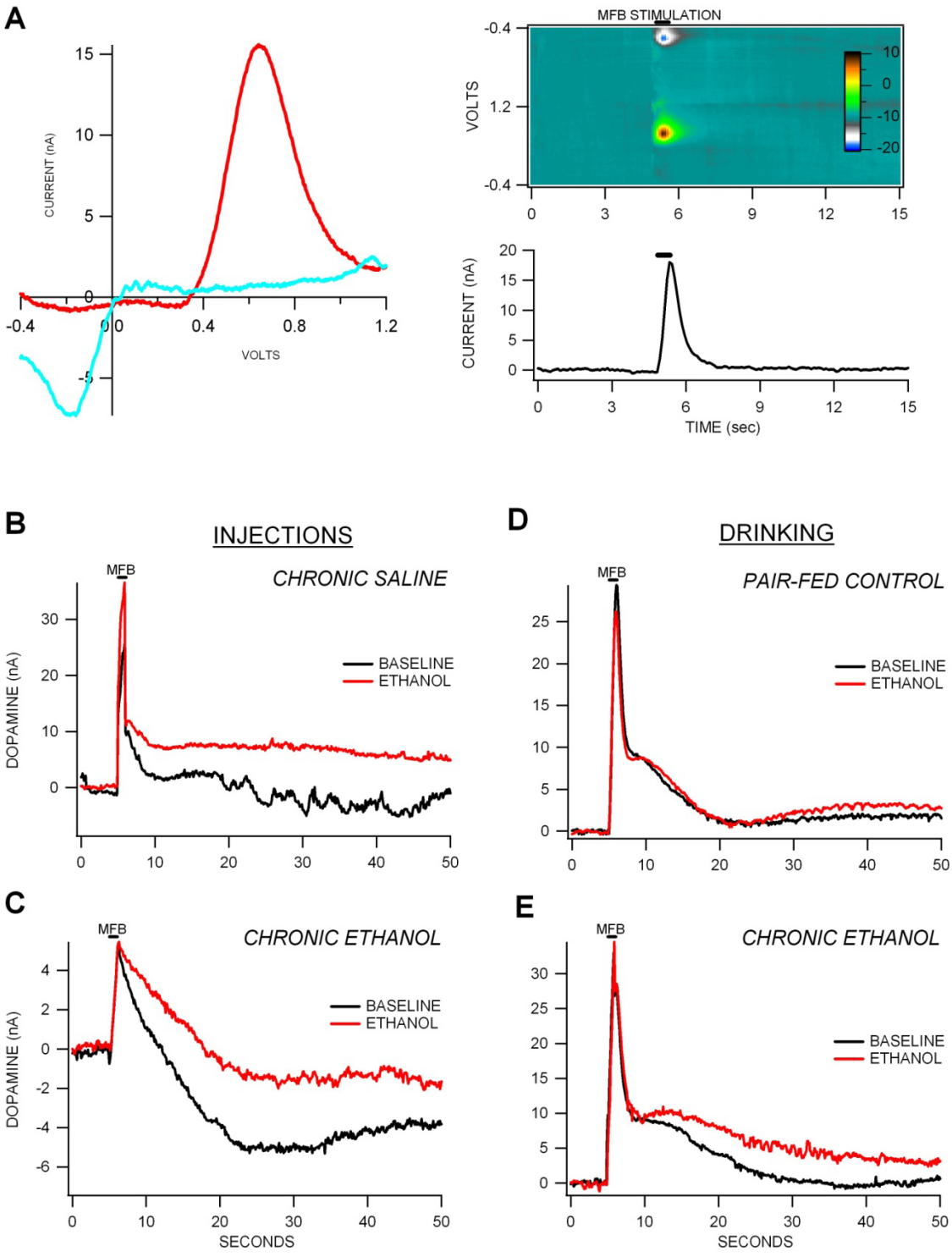


***Figure 10*. Effects of non-contingent and contingent ethanol on dopamine release via fast scan cyclic voltammetry.** (A) These graphs illustrate how dopamine is measured. The left graph shows the current

obtained by an ascending and descending voltage ramp during stimulation of the medial forebrain bundle (MFB). Note the oxidation that occurs during the ascending voltage ramp (red) and the reduction that occurs during the descending voltage ramp (blue). The graph at top right shows a density color plot showing oxidation and reduction currents during MFB stimulation. In other words, this is a plot of voltammograms over time before and after MFB stimulation. The graph underneath shows the current response obtained from the voltammogram that corresponds to dopamine (based on a calibration current). The scale is normalized in these two graphs so that comparisons can be made. (B,C) Effects of acute ethanol (2.0 g/kg) on the averaged dopamine signal obtained in animals chronically treated with saline (B) or ethanol (C) injections. Note that the baseline dopamine signal in chronic ethanol treated rats was significantly lower than that obtained in the chronic saline treated rats. However, the effects of an acute challenge of ethanol were fairly similar, characterized by prolonged kinetics typical of ethanol. (D,E) Effects of acute ethanol on the averaged dopamine signal obtained in rats chronically drinking ethanol vs. their pair-fed controls. Note that there was only a small difference between pair-fed and chronic ethanol treated rats.

Discussion

Results in the experiments have shown that VTA GABA neuron activity was affected by HT7 electoacupuncture, which consistently produced a biphasic modulation of firing rate with a transient enhancement followed by a more prolonged inhibition, recovering in 5 min. Tail stimulation, however, yielded only a transient enhancement of the firing rate, which typically occurs in association with generalized sensory stimulation, as demonstrated in our previous study (Ludlow, et al., 2009). Most importantly, HT7 inhibition of VTA GABA neuron firing rate was blocked by naloxone, a non-selective MOR antagonist, indicating that stimulation of this specific acupuncture point activates opioidergic inputs to VTA GABA neurons, perhaps from the

acupuncture-sensitive arcuate nucleus of hypothalamus (Q. Wang, et al., 1990a, 1990b). Thus, as hypothesized, acupuncture at a specific site of heart channel, HT7 seems to be involved in releasing endogenous opioids in the VTA, which may lead to activate a particular OR, MOR. The activation of this MOR inhibits consequently the GABA neuron activity through hyperpolarizing. In addition, the typical inhibition of VTA GABA neurons produced by an intoxicating dose of ethanol (Gallegos, et al., 1999; Ludlow, et al., 2009; S. C. Steffensen, et al., 2009; Stobbs, et al., 2004) was unaffected by continuous Tail stimulation. In contrast, the same dose level of ethanol excited VTA GABA neurons firing during continuous HT7 stimulation, suggesting that HT7 acupuncture directly reverses ethanol's inhibitory effects on VTA GABA neurons, or indirectly suppresses through interaction with synaptic inputs to the neurons either in the VTA or NAcc. Because VTA GABA neurons express MORs and are inhibited by opioids (S. C. Steffensen, et al., 2006), and acupuncture enhances opioid-mediated transmission in the arcuate nucleus, which projects to the VTA, we speculated that OR modulation by HT7 electroacupuncture might be contributing to its ability to reduce ethanol inhibition of VTA GABA neurons. Accordingly, systemic administration of naloxone blocked not only HT7 inhibition of GABA neuron activity but also HT7 block of ethanol inhibition of VTA GABA neuron activity. Initially, it seemed counterintuitive, as both HT7 and ethanol inhibit VTA GABA neuron activity. However, there was a slight difference between ethanol inhibition and that of HT7 stimulation. In other words, when applied alone, whereas ethanol inhibition of GABA neuron activity was prolonged, OR-sensitive inhibition generated by HT7 stimulation was transient, and might be subject to concomitant involvement of DORs in accumbal GABA terminals to VTA GABA neurons. Although naloxone blocked the effects of HT7 acupuncture as well as its effects on ethanol inhibition of VTA GABA neuron firing rate, the mechanism of

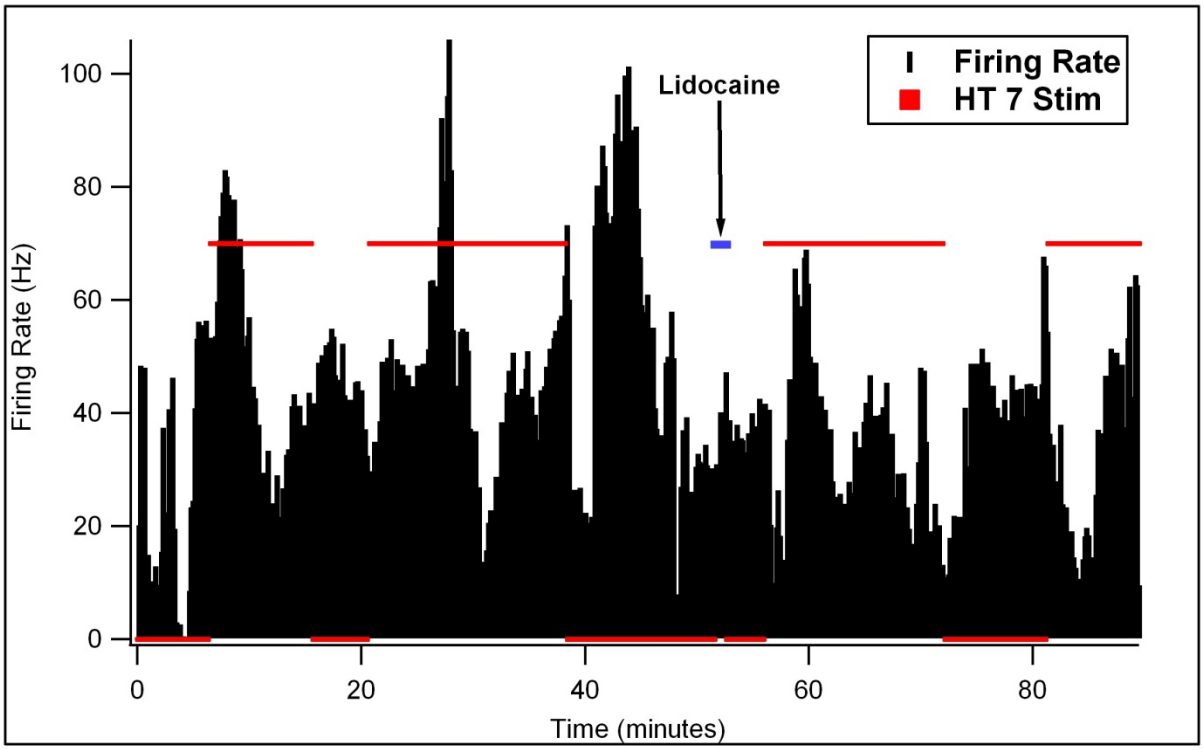
action occurring between acupuncture and MOR is still uncertain, given that naloxone is a non-selective OR antagonist. Although MOR is sensitive to naloxone, it likely also blocks κ- and δ-OR at the high doses, which is used to block HT7 inhibition of VTA GABA neuron firing rate. Also, the roles of ORs such as Kappa-opioid receptor (KOR) and Delta-opioid receptor (DOR) other than MORs cannot be ruled out. Mostly, the acupuncture's effects might be due to a combination of actions on MORs and/or DORs in the VTA, NAcc, or accumbal GABA inputs to the VTA. Accumbal GABAergic inhibitory input to VTA GABA neurons has been known to be formidable, and a combined effect on MORs and DORs is likely to occur, as a recent report has implicated DORs on accumbal GABA terminals of VTA GABA neurons in ethanol consumption (Margolis, Fields, Hjelmstad, & Mitchell, 2008). Paradoxically and unexpectedly, naloxone, a MOR antagonist, when employed with HT7 stimulation had no effect or slightly enhanced ethanol inhibition of VTA GABA neuron firing rates. It may be debatable and also a new paradigm because it has been demonstrated in many other studies including human research that along with a strong non-selective OR antagonist naltrexone, naloxone has shown to consistently inhibit ethanol consumption as well as ethanol-induced place preference. But, on the other hand, we need to call our attention to an important fact that in many studies, only observation of behavioral reinforcing effects caused by chronic ethanol self-administration has been made to demonstrate the suppressive effects of OR antagonists, as opposed to our present experiments conducted in acute ethanol administration condition to evaluate the effects of OR antagonists on the ethanol inhibition of GABA neuron activity in the VTA. Indeed, it is a well-known fact that stimulation of MOR and DOR was linked to the positive reinforcing effects of ethanol, whereas stimulation of the KOR mediated the aversive aspects of ethanol action (Higley & Kiefer, 2006). Thus, in order to clarify the role of DORs in ethanol effects on VTA GABA neuron activity, we

compared the effect of naloxone with that of the DOR antagonist, naltrindole. Unlike naloxone, naltrindole eliminated the inhibitory effect of ethanol on VTA GABA neuron firing rates, suggesting the involvement of DORs in ethanol effects in the VTA. It appears that the DOR antagonist, naltrindole plays a similar role in blocking ethanol inhibition of VTA GABA activity to that HT7 acupuncture stimulation had. However, supplemental in-depth investigation on the opioid in the VTA and their receptors in future studies is inevitably required to determine if the effect is on DORs on accumbal GABAergic terminals of VTA GABA neurons (Margolis, et al., 2008) or on DORs in the NAcc (Lu, et al., 1998; Mansour, Watson, & Akil, 1995; S. Weiner, Shaikh, Shaikh, & Siegel, 1991). We tried to complement the role of the selective OR antagoinist, naltrindole in reduction of ethanol effects on ethanol-induced behaviors in rats. In the experiments conducted by our research collaborators at Daegu Haany University in Korea, the effects of acupuncture on animal behaviors after ethanol administration were demonstrated. The stimulation at HT7, but neither at PC6 nor at Tail, significantly decreased ethanol-reinforced responding, showing that this effect is specifically limited to the particular point, HT7. As insertion of needles into acupuncture points on the forepaw has been known to give rise to motor impairment, we attempted to control for the possibility of generalized effects of acupuncture on response rates by monitoring sucrose-reinforced responding. In the result, acupuncture did not influence the responding with sucrose pellets, implying that acupuncture may exert an inhibitory effect on ethanol self-administration by reducing motivational, rather than performance-related, influences. As suggested by several studies previously, ethanol enhancement of DA neurotransmission in the mesolimbic system has long been associated with positive reinforcement contributing to ethanol-seeking behaviors (Czachowski, Chappell, & Samson, 2001; Melendez, et al., 2002; Rodd, et al., 2004; Walker & Ettenberg, 2007; F. Weiss, Lorang,

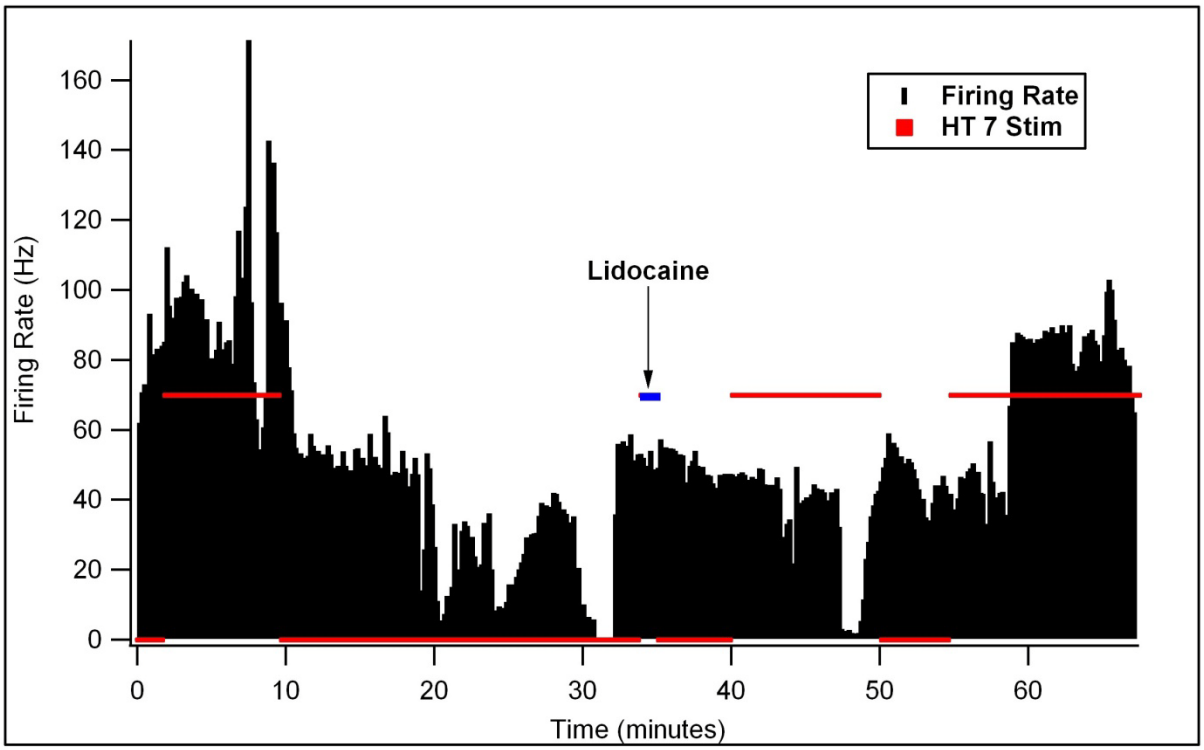
Bloom, & Koob, 1993). In a recent study, we demonstrated that acupuncture at HT7 significantly inhibited ethanol-induced DA release in the NAcc through the $GABA_B$ receptor (Yoon, et al., 2004). Based on these observations, our results indicate that the reduction of ethanol-reinforced response by acupuncture at HT7 is most likely mediated via a suppression of DAergic activity in the NAcc.

In many studies, acupuncture has been shown to activate opioidergic fibers originating in the arcuate nucleus that extend to both the VTA and the NAcc. Specifically, the opioid such as endorphine released from the fibers excites MORs in the VTA, which results in inhibition of the GABA neuron. However, with a few critical electrophysiological and pharmacological experiments of our preliminary studies, it still seemed unclear whether acupuncture inhibits VTA GABA neuron activity directly through activation of MORs on VTA GABA neurons by opioidergic inputs or indirectly through activation of other ORs on NAcc neurons that inhibit VTA GABA neurons. Furthermore, the inhibition didn't last more than 5-10 min, suggesting that there should be some other effects on the inhibition of VTA GABA neurons either in the VTA or from the NAcc. In particular, as shown in our previous study (S. C. Steffensen, et al., 2006), it is also known that systemic local injection of such MOR agonists as DAMGO or heroin in the VTA inhibits GABA neuron firing rates, presumably through activation of MORs on VTA GABA neurons. But, when combined with subsequent administration of a moderate dose of MOR antagonist, naloxone, the inhibition incurred by MOR agonist was significantly blocked, recovering the initial firing rate. Also, we found an interesting fact in this study that opiates might affect the opioid-sensitive NAcc input to VTA GABA neuron. Lidocaine, a local anesthetic, infused ipsilaterally to inactivate the NAcc neurons, showed a remarkable effect by increasing VTA GABA firing rates. Thus, expecting the lidocaine's effect on the increase of

GABA neuron firing rate as well as its effect on HT7 inhibition of VTA GABA neuron activity, I suggested the inactivation of the NAcc. With a view to obtaining the similar results from application of HT7 stimulation rather than opioids, we investigated the sensitivity of VTA GABA neurons in response to 5 min HT7 stimulation and repeated in 10 min to make sure that it was consistent. After placing the cannula in the NAcc, we injected 1 μL of lidocaine and retested with HT7 acupuncture stimulation in 1 min. Of the three neurons, we tested two neurons sensitive to HT7 and the inactivation of NAcc seemed to block HT7 inhibitory effect on the firing rate rather than directly to influence the VTA GABA neuron firing rate. But, due to inconsistent mediating effects of lidocaine **(**see Figure 11**)** and a relatively small number of acupuncture stimulation -sensitive neurons tested, it was not possible to assert how acupuncture modulates specific opioid receptors in either VTA or NAcc to influence ethanol inhibition after the NAcc was inactivated. With all the degree of complexity and difficulty in the experiments, it is still worth replicating in the future with an adequate number of GABA neurons sensitive to acupuncture stimulation because the GABAergic input from the NAcc may be modulated by opioids and could be a critical regulator of VTA GABA neuron activity.



HT 7 activation without lidocaine block



HT 7 activation with lidocaine block

***Figure 11*. Effects of inactivation of the NAcc by lidocaine on HT7 modulation of VTA GABA firing rate.** This figure shows that the inhibition of VTA GABA firing rate by HT7 acupuncture simulation with or without lidocaine block of NAcc. As seen in the figure, when the NAcc is not inactivated by the local anesthetic, lidocaine, the firing rate was consistently inhibited (above). However, the VTA GABA neurons seemed to increase in their firing rate with the block of the NAcc (below).

Also, I proposed to evaluate the activity of VTA GABA and DA neurons in the NAcc affected by HT7 acupuncture stimulation in both chronic ethanol dependent and independent condition. In other words, we planned to measure the effects of chronic ethanol injection and consumption on the DA signals, respectively. First, in an independent condition, we tried to ensure whether the ethanol-containing diet would be practically useful in inducing the rats to consume ethanol continuously without affecting their health, considering the tendency of disinclination toward ethanol. Contrary to our expectation, however, the rats seemed to adapt to the new liquid food successfully, increasing their body weights consistently. In the beginning, the ethanol- containing diet treated rats showed their dislike for the new diet by burying it with beddings in the cages for a while but soon started to increase their intake. We recorded how much they consumed the diet daily, increasing the concentration of ethanol in the diet incrementally for the ethanol rats to ensure there was no deleterious impact on their health. However, the results in the experiment showed that HT7 stimulation seems to have little effect on DA neuron release following chronic ethanol in non-dependent condition, compared to pair-fed controls. When measured prior to HT7 electrical stimulation, the baseline of DA release in ethanol treated rats was a little higher than that in pair-fed control rats. We were surprised to learn that the rats with chronic ethanol in dependent condition later revealed the opposite results, showing relatively small DA signals compared to control rats given with saline. However, we

had inconsistent data in DA levels for the experiment even after challenging with an acute ethanol intraperitoneal administration. In a previous study on ethanol effects on DA release, even a single dose of ethanol at low or moderate dose would decrease evoked DA release in many areas including caudate putamen, substantia nigra of ambulatory male rates as measured by FSCV. Besides, it was due not to increase in DA uptake rates but to a direct suppression of DA release. In general, the ethanol effects on DA release in the brain are believed to accompany the interaction with $GABA_A$ receptors that decrease the firing rates of GABA neurons in the area and it should be also applied to the rewarding circuits, VTA and NAcc. However, the ethanol inhibits GABA neuron firing in the VTA with the acute administration of a moderate dose, which is an opposite effect. In this new paradigm, we applied a different method of administration of ethanol to the NAcc followed by electric stimulation of MFB to see how DA levels of concentration are reacted before evaluating the effect of electric stimulation by acupuncture. With a clear and significant difference in DA levels, we would have been able to repeat the experiment with more rats, along with physiological surgery of brain tissue punches of the VTA, NAcc, and cortex for quantitative reverse transcription-polymerase chain reaction (RT-PCR). Consequently, we decided not to continue this novel experiment. We supposed that it might be problematic for several different reasons including positioning electrode and HT7 acupuncture points. There might have been some factors affecting sensitivity of electrodes with the voltammetry method. The size of carbon fiber may determine the number of sites being sampled for DA release or uptake. The other potential factor to consider in measuring subsecond scale DA concentration might be the method of implantation of electrodes in the brain. In other words, with more frequent use of the electrode in the surgery, it usually loses its sensitivity to DA oxidation and may have a slower time response, which would be critical. Also, we might have to consider

taking account that DA release can be modulated by activating receptors on DA terminals where D2 autoreceptors that inhibit the release of DA are expressed (Fields, Hjelmstad, Margolis, & Nicola, 2007). Then, we should investigate how they are affected by the chronic ethanol self-administration or injection. Nevertheless, we found in this experiment at least that there was a small, but not statistically significant difference in DA neurons release between ethanol diet treated rats and their pair-fed rats.

As mentioned, the DA levels showed a different pattern in this dependent situation. That is, the chronic ethanol injection group showed relatively low level of DA compared with their pair-fed group. In fact, it is in agreement with the previous studies, demonstrating that the VTA GABA neurons show tolerance to the chronic ethanol, resulting in elevating inhibition of DA neurons activities in the NAcc. We performed this experiment with a different method, FSCV, instead of microdialysis used by our research collaborators in Korea who have already shown comparable data. In general, the microdialysis has limitations in evaluating whether DA depletion in the NAcc may be due to its adaption to DA uptake, or measuring DA release on a subsecond scale. Unfortunately, we had also sick rats with regular injection of ethanol in this dependent condition. Two rats in the ethanol injection group had either internal bleeding or massive diarrhea that eventually prevented them from reaching electrophysiological surgery procedure. Their intraperitoneal cavities seemed inflated enormously with persistent injection of ethanol. The amount (4.4 - 4.7 ml) administered in a 5 ml syringe twice a day seemed overwhelmingly unbearable. For future replicating studies, it would be a good idea to assess the blood alcohol level prior to preparation of ethanol injection, as demonstrated in a recent study (Ludlow, et al., 2009). Concerning the results shown in this experiment, the effects of a challenge of acute ethanol were fairly similar, characterized by prolonged kinetics typical of

ethanol. In this experiment, we failed to prove a clear modulation by HT7 stimulation of DA release or uptake in the NAcc as opposed to our expectation that electroacupuncture at HT7 may incur some kind of modulation to the region through its opioidergic input from arcuate nucleus of hypothalamus. But, on the basis of preliminary relevant studies, it seems that HT7 electroacupuncture plays a normalizing effect on DA levels in ethanol treated group as it blocks the inhibition of acute ethanol in suppressing VTA GABA neuron firing following its adaptation to chronic injection of ethanol. On the other hand, the DA levels in the saline treated group didn't seem affected by HT7 electrical stimulation, showing inconsistent change of the DA concentration levels. Despite the insight obtained by studying VTA GABA neurons and DA release during adaptation to chronic ethanol rats, we couldn't validate the results due to the small number of rats used for these experiments. Moreover, there were some inevitable limitations in the experiments. There were erratic occurrences of single-unit recordings, including the correlative nature of the data and its interpretation. There were also unpredictable events of the DA electrochemistry method in considering the fact that ethanol decreases DA FSCV, which is the opposite of its effects by DA microdialysis. However, single-unit recordings of critical neural substrates involved in drug reward and measuring DA neurotransmission will go far in aiding our understanding the neurobiology of the intoxicating and rewarding properties of alcohol.

Actually, the results that have been acquired in genetic models of high preference for ethanol support the view that its intake relies on the activity of the endogenous opioid reward system, and that ethanol consumption may serve to compensate for inherent deficits in this system (Herz, 1997). This hypothetical model proposes that reward, including ethanol reward, results from activation of MORs in the VTA (see Figure 2) and/or DORs in the NAcc. Both the VTA and NAcc nuclei are targets of endogenous β-endorphin from the arcuate nucleus, and

MORs and DORs in these nuclei appear to be implicated in ethanol consumption, as their selective antagonists and agonists decrease or increase ethanol intake, respectively (Herz, 1997). How might ethanol and opioids interact in the VTA? First, they have similar effects on VTA GABA neurons. We and others have shown that ethanol and MOR agonists inhibit VTA GABA neuron firing rates (Gallegos, et al., 1999; S.W. Johnson & R.A. North, 1992; Ludlow, et al., 2009; Margolis, Lock, Hjelmstad, & Fields, 2006; Stobbs, et al., 2004; Xiao & Ye, 2008; Xiao, Zhang, Krnjevic, & Ye, 2007), suppress GABA inhibition of VTA DA neurons (Xiao & Ye, 2008; Xiao, et al., 2007), and excite VTA DA neurons (Gessa, Muntoni, Collu, Vargiu, & Mereu, 1985; S.W. Johnson & R.A. North, 1992; Margolis, Hjelmstad, Bonci, & Fields, 2003; Xiao, et al., 2007), likely via disinhibition (S.W. Johnson & R.A. North, 1992; Xiao & Ye, 2008; Xiao, et al., 2007). Second, ethanol potentiates GABA IPSCs in VTA DA neurons in the presence of saturating concentrations of MOR agonists (Xiao, et al., 2007). Contrary to the typical potentiating effects of ethanol on GABA transmission in other brain areas (G. R. Siggins, Roberto, & Nie, 2005; J. L. Weiner & Valenzuela, 2006), ethanol inhibits GABA neurons in the VTA and consequently inhibits GABA IPSCs in VTA DA neurons (Xiao, et al., 2007). Because VTA GABA neurons are the primary source of inhibitory input to VTA DA neurons, ethanol inhibition of GABA IPSCs appears to dominate over the potentiating effect of ethanol on other GABAergic or opioidergic inputs to VTA neurons. Third, ethanol enhances the release of β-endorphin (Herz, 1997; Marinelli, Quirion, & Gianoulakis, 2004; Stein, 1993), which might activate MORs on VTA GABA neurons. Thus, acupuncture's effects on VTA GABA neurons might result from combined effects on opiodergic actions on VTA GABA neurons via MORs or on accumbal GABA input to VTA GABA neurons via DORs. In support of the latter, a recent study has shown that activation of DORs in the VTA decreases ethanol intake in rats (Margolis,

et al., 2008). This study implicates presynaptic modulation of GABA release in the VTA by DOR since a DOR agonist, DPDPE inhibits evoked, spontaneous, and mini GABA IPSCs. Accordingly, ethanol consumption up-regulates DORs, but down-regulates MORs in the VTA (Mendez, Leriche, & Calva, 2001). Although many studies have reported a role of both MORs and DORs in regulating ethanol intake, the evidence for the involvement of ORs in the VTA remains complex and controversial. In some previous studies, the effect of DOR antagonist, naltrindole was compared with that of a non-specific OR antagonist. Although reducing the ethanol consumption in alcohol-preferring rats, the inhibitory effect of naltrindole was not as strong as that of naltrexone. Moreover, they argued the ineffectiveness of higher dose of naltrindole than a specific amount, 8 mg/kg (Higley & Kiefer, 2006). However, the finding is not consistent with the result of our present experiment in which the effect of 15 mg/kg naltrindole on ethanol-induced responding was demonstrated. But, in a previous study evaluating the differential effects by selective OR antagonists on alcohol-seeking induced by discrete cues and context, CTOP, a MOR antagonist didn't have an inhibitory effect on reinstating alcohol seeking, while intraperitoneal injection of naltrindole (0-15 mg/kg) reduced significantly. In addition, it showed that the doses of more than 10 mg/kg naltrindole produced the locomotor impairment (Marinelli, et al., 2009). It is a little surprising in that the high doses caused motor impairment that didn't occur in our present study. We supposed that it might be a result of deficiency of previous self-administration training such as sucrose fading procedure. Our findings provided some insight into the role of MOR and DOR involvement in ethanol effects and acupuncture modulation of ethanol effects. We have shown that both acupuncture and naltrindole reverse ethanol inhibition of VTA GABA neurons and reduce ethanol self-administration without affecting behavioral responses. We may propose that ethanol and acupuncture have an impact

on opioidergic mechanisms in both the VTA and NAcc, but that the effect of ethanol on VTA GABA neurons is predominantly mediated via DORs and not MORs. Possibly, acupuncture may reduce DOR transmission, as acupuncture and naltrindole have nearly identical influence on ethanol effects on VTA GABA neurons and on ethanol consumption. It has been recently proposed a hypothetical model that might explain how HT7 acupuncture might inhibit VTA GABA neuron activity (Yang, et al., 2008). Acupuncture or electroacupuncture may activate the ulnar nerve, which lies adjacent to the HT7 acupuncture point (Peuker & Cummings, 2003). Subsequently, sensory stimulation activates enkephalinergic and β-endorphinergic neurons in the arcuate nucleus of the hypothalamus (Q. Wang, et al., 1990a, 1990b), and endorphinergic fibers projecting from the arcuate nucleus can in turn activate MORs on VTA GABA neurons (Mansour, et al., 1988). The inhibition of the VTA GABA neuron firing rate by HT7 stimulation recovered in approximately 5 min, suggesting that opioidergic processes are being recruited to counteract the direct effects of opioid inhibition on VTA GABA neurons. Indeed, our previous studies have indicated that opioid modulation of VTA GABA neurons is complex, involving direct and indirect effects. For example, opioid inhibition of VTA GABA neurons involves not only direct effects on MORs onto GABA neuron (via *in situ* MOR agonist activation), but also latent indirect effects on accumbal GABAergic inhibition of VTA GABA neurons (S. C. Steffensen, et al., 2006), and perhaps influenced by DOR modulation of accumbal GABAergic inhibition on VTA GABA neurons (Margolis, et al., 2008). Notwithstanding the complexities of opioid effects on VTA GABA neurons, HT7 inhibition of their firing rate through MOR activation might lead to the short-term enhancement of mesolimbic DA release via disinhibition of VTA DA neurons. Taken together, these findings support the hypothesis that opioid-mediated ethanol inhibition of GABAergic synaptic transmission to VTA DA neurons may have a critical

role in ethanol reward. Moreover, they support the emerging view in the alcohol literature of an interaction between endogenous opioids and ethanol in the mesolimbic system (Herz, 1997), and the current clinical use of opioid antagonists to prevent relapse in alcoholics (Oswald & Wand, 2004). The mesolimbic system is closely connected to the reinforcing and locomotor activity stimulating effects of drug of abuse, including alcohol. Repeated administration of alcohol eventually produces behavioral sensitization which is associated with an increase in DA release in the NAcc. This sensitization is believed to be implicated in the development of addictive symptoms. This study shows that acupuncture treatment at a specific site may significantly reduce the expected increase in alcohol-induced responding, including relapse to alcohol addictive behaviors and inhibition of the dominant inhibitory neuron in the motivated and rewarding circuit in the brain. Based on these results, I suggest that acupuncture could play a significant role in suppressing the reinforcing effects of alcohol. Furthermore, as seen in the present study, it can also make a difference in the treatment of alcohol abuse or alcoholism by providing an effective medical therapy, along with OR antagonists in clinical fields in the future.

Yet, there are some issues to be dealt with before our future replication on the studies that were proposed but has not been completed. First, in the Liquid diet, regardless of consistent eating habits even with a 5% ethanol concentration, the aversive behavior of rats such as burying the diet with bedding might have affected the consumption. And in chronic ethanol injection treatment, sufficient knowledge about Blood Alcohol Level (BAL) from relevant or similar studies conducted previously could prevent the loss of rats prior to electrophysiological experiments. Besides, another run of chronic injection experiment in pairs of rats seemed helpful in obtaining a more robust result about the significance of acupuncture stimulation on chronic ethanol effects. With regard to the positioning of a specific acupuncture point, HT7 in the

experiment, a more careful attention in stimulating seems required because variance can make an undesirable or unexpected result. Also, the change of the order between HT7 simulation and ethanol or OR antagonists administration in future studies to strengthen our hypothesis might be considered. Even different doses of those drugs could be an option for consideration because the effects of opioid receptor agonist or antagonist on ethanol-induced behaviors are still indecisive and controversial in many studies. In this study, we discovered that two endogenous opioids and their receptors, Mu-OR and Delta-OR play a significant role in reward and addictive properties of ethanol because the reinforcing properties of MOR and DOR ligands occur with activation of the mesolimbic DA system (Herz, 1997). However, consideration of mediating effects of Kappa-OR in the opioid system on the rewarding and addictive process produced by ethanol would be necessary because it is well known that there is also interaction between dynorphin, Kappa endogenous ligand, and reward. Then, it would be a good opportunity to replicate with application of acupuncture stimulation and observe whether it would still show its critical effect.

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