NEURONAL ADAPTATIONS IN THE NUCLEUS ACCUMBENS CORE

UNDER ACUTE AND CHRONIC EXPOSURE TO CANNABINOIDS

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Sung Ha Lee

NEURONAL ADAPTATIONS IN THE NUCLEUS ACCUMBENS CORE UNDER

ACUTE AND CHRONIC EXPOSURE TO CANNABINOIDS

Cannabis, known as marijuana, is the most commonly used illicit drug in the United States, but we have limited knowledge about its effects on the brain, particularly the reward circuitry. Cannabinoids, the psychoactive ingredient of cannabis, activate cannabinoid receptors in the mesolimbic area, resulting in increased dopamine transmission in the nucleus accumbens (NAc). This effect is believed to enhance goal-directed behavioral responses, including the motivation to obtain natural and drug rewards, but NAc signaling under cannabinoid exposure remain largely unknown. To address this gap, this dissertation work examines two main signaling changes in the NAc core: neuronal activities and dopamine dynamics.

Since cannabis derivatives are usually used for a prolonged time, ongoing changes in the NAc core were investigated in response to acute and repeated exposure of cannabinoids. Therefore, in Experiment 1, NAc neuronal signaling was obtained on initial and repeated exposure (seven daily injections) of a cannabinoid receptor agonist, CP55,940 (0.2 mg/kg or 0.4 mg/kg) in male Sprague–Dawley rats using an *in vivo* electrophysiology technique. The overall effect of CP55,940 on CB1/2 receptor activation acutely inhibited NAc neuron

activity and reduced correlated neuronal activity/burst firing, and these effects lasted for the seven days of injections. This result suggests that cannabinoids reduce neuronal signaling and disrupt functional communication in the NAc core for a prolonged period. However, cannabinoid increased the theta power of local field potentials after acute CP55,940 injection but repeated treatment failed to maintain this effect.

In Experiment 2, the electrically evoked dopamine overflow was collected using fast scan cyclic voltammetry (FSCV). Using kinetic analysis, dopamine release and reuptake were assessed immediately following one or seven daily injections of CP55,940 (0.2 mg/kg, i.p.) or a vehicle. A single injection of CP55,940 increased the stimulation-evoked dopamine concentration without altering dopamine reuptake. However, repeated CP55,940 exposure led to a similar level of dopamine concentration as the chronic vehicle treatment.

The sustained neuronal signaling but altered dopamine dynamics in the NAc core after repeated cannabinoid exposure suggest separate mechanisms in the development of tolerance. As the present results indicate, altered signaling of the NAc core could provide evidence of changes in motivational states that, in turn, may play a role in changing reward-related behaviors.

Table of contents

Introduction

In Western countries, marijuana, derived from the plant *cannabis savita*, is the most widely used illicit drug for recreational use (Cadoni, Pisanu, Solinas, & Acquas, 2001). Human marijuana users have generally reported that smoking marijuana produces relaxation, a sense of euphoria and a feeling of well-being (D'Souza et al., 2008). The well-known primary psychoactive component of cannabis is Δ9-tetrahydrocannabinol (THC), a partial agonist of cannabinoid receptor 1 (CB1). Both synthetic cannabinoid receptor agonists (e.g., WIN 55,212 and CP55,940) and THC are known to excite CB1 receptors in the brain. Like humans, animals also appear to find cannabinoid agonists to be rewarding; animals prefer cannabinoids over saline in conditioned place preference (Braida, Pozzi, Parolaro, & Sala, 2001b), cannabinoids lower intracranial self stimulation (ISCC) (Gardner et al. 1988;Negus & Miller, 2014), and animals are able to self administer cannabinoids (Tanda & Goldberg, 2003).

The rewarding effect of cannabinoids comes from activation of the brain reward circuitry. Addictive drugs including cannabis are characterized by increasing extracellular dopamine in the synapse of dopaminergic neurons projected from ventral tegmental area (VTA) (Di Chiara, 1997). The axons from dopaminergic neurons project primarily into the nucleus accumbens (NAc) and the medial prefrontal cortex (PFC) (Riegel & Lupica, 2004) and form the mesolimbic

pathway and mesocortical pathway, respectively. Phasic dopamine release in the reward circuit including meso- and corticolimbic regions encodes natural rewards including food, sex, and social interactions (Kelley & Berridge, 2002) as well as addiction to drugs of abuse (Nestler, 2005).
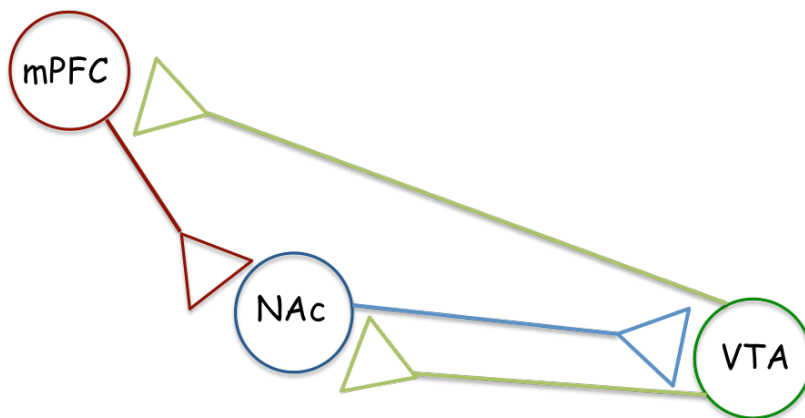
Figure 1. Brain reward circuitry. A simplified diagram of the major dopaminergic (green), glutamatergic (red), and GABAergic (blue) connections among the ventral tegmental area (VTA), nucleus accumbens (NAc) and medial prefrontal cortex (mPFC).

Endocannabinoid signals in the mesolimbic pathway

Cannabinoids primarily act on the endocannabinoid system, which is comprised of cannabinoid receptors and endogenous cannabinoids. The two best-known endogenous ligands for cannabinoid receptors are anadamide (AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Sugiura, Kodaka, Kondo, & Tonegawa, 1996). Both are derived from arachidonic acid in the cell membrane and released "upon demand" (stimulation) without being stored in vesicles. The

first cannabinoid receptor, called CB1, was sequenced and cloned (Matsuda, Lolait, Brownstein, & Young, 1990; Matusda, Lolai, Brownstein, & Young, 1990) and three years later, CB2 was discovered (Matsuda, Bonner, & Lolait, 1993). CB1 and CB2 receptors are metabotropic receptors coupled to $G_{i/o}$ proteins; once activated, they inhibit adenylyl cyclase, close Ca^{2+} channels, open K^+ channels, and stimulate kinases, which phosphorylate tyrosine, serine, and threonine residues on various proteins (Solinas, Goldberg, & Piomelli, 2008).

The endocannabinoid system is crucial in dopamine signaling in motivated behavior (Oleson et al., 2012; Solinas et al., 2008). CB1 receptor activation mediates transient increases of dopamine in the NAc during reward seeking behavior (Oleson et al., 2012; Oleson & Cheer, 2012). Also, blocking CB1 receptor attenuate drug seeking behavior not only elicited by cannabinoid agonist but also other abused drugs (Hernandez & Cheer, 2011). Also, increasing endocannabinoid tones by inhibiting degradation enhance dopamine level in the NAc as well as reward seeking behavior (Oleson et al., 2012). Thus, endocannabinoid signal in the VTA is essential to modulate dopamine release in coding incentive motivation.

CB1 receptors located on the presynaptic axonal terminal are well-characterized to suppress the release of neurotransmitters such as glutamate and GABA (Freund, Katona, & Piomelli, 2003). Disinhibition of dopaminergic cells via CB1

3

receptor activation in the VTA is known as the core mechanism of the cannabinoid-induced modulation in the reward pathway. In other words, CB1 receptor activation reduces the release of the inhibitory neurotransmitter GABA onto VTA neurons, thus facilitating the burst firing of dopaminergic cells, which subsequently results in dopamine release in the PFC and NAc (French, 1997).

Cannabinoid-induced effects on the mesolimbic pathway

1. Dopamine signaling in the NAc

It has been established that cannabinoids increase dopamine release in the axon terminal of VTA dopamine neurons, as other abused drugs do. First, using microdialysis, Chen and colleagues reported that systemic administration of THC in freely moving animals increased the dopamine concentration in the NAc (Chen et al., 1990). A few years later, this study was successfully replicated to show that THC, as well as WIN 55,212, increased dopamine release in NAc and that this effect was blocked by the CB1 receptor antagonist, SR141716 (Tanda, Pontieri, & Di Chiara, 1997). Moreover, using a "real time" technique called fast scan cyclic voltammetry (FSCV), it was confirmed that cannabinoids enhance subsecond dopamine transient in the NAc of freely moving animals (Cheer, Wassum, Heien, Phillips, & Wightman, 2004).

The possible underlying mechanism of this increase of NAc dopamine would be an increase in the burst firing of VTA neurons by exogenous cannabinoids. The VTA, located in the midbrain, is composed of dopaminergic cells and non-dopaminergic (most of them are GABAergic) cells (Lupica, Riegel, & Hoffman, 2004). VTA dopaminergic neurons normally fire at 5-10 Hz (pace-maker firing) in freely moving rats (Kiyatkin & Rebec, 1997), but in response to salient stimuli, the firing pattern becomes bursts of about 20 Hz (Hyland, Reynolds, Hay, Perk, & Miller, 2002). VTA dopaminergic neurons are reported to show burst activity in response to rewards, including abused drugs, which results in phasic dopamine release in the axonal synapse (Schultz, 2007).

2. VTA dopaminergic neurons

CB1 receptors have been identified in both glutamatergic and GABAergic synapses in the VTA (Mackie, 2005; Mátyás et al., 2008). The anatomical studies suggests that not only can endogenous cannabinoids modulate the reward pathway, but also that exogenous cannabinoids can directly modulate GABA or glutamate release on VTA dopaminergic neurons. Along with the presence of CB1 receptor in the VTA, cannabinoids also modulate VTA dopaminergic function via indirect mechanism (Wenzel & Cheer, 2014). The VTA receives input from the various nuclei involved in integrating sensory information and motor output. Among these, three major glutamate afferents are located in the medial

5

prefrontal cortex, the pedunculopontine region, and the subthalamic nucleus (Melis, 2004). Also, the VTA receives major GABAergic inputs from NAc, mesopontine rostromedial tegmental nucleus (RMTg), and ventral pallidum (Lecca, Melis, Luchicchi, Ennas, Castelli, Muntoni, et al., 2011a).

Endogenous as well as exogenous cannabinoids mediate the presynaptic inhibition of glutamate transmission in the VTA (Melis, 2004); WIN55,212 reduced both NMDA and AMPA currents in the slice preparation of VTA dopaminergic neurons. The endogenous cannabinoid 2-AG also reduces the glutamate mediated long-term potentiation (LTP) of VTA DA neurons (Kortleven, Fasano, & Thibault, 2011). Thus, cannabinoid receptor activation reduces glutamate transmission possibly via presynaptic inhibition of glutamate release. However, suppression of glutamate release on VTA dopaminergic neurons in response to cannabinoids might contradict previous studies showing increased DA release after cannabinoid administration.

CB1 receptor activity is highly linked to VTA dopaminergic neuronal firing via inhibitory afferents to these cells. The mesopontine rostromedial tegmental nucleus (RMTg), also known as a tail of the VTA, provides the major GABAergic input to the VTA (Lecca, Melis, Luchicchi, Ennas, Castelli, Muntoni, et al., 2011a). Cannabinoids significantly suppress RMTg activity, which is reversed by the CB1 antagonist, SR141716 (Lecca, Melis, Luchicchi, Ennas, Castelli, Muntoni, et al.,

2011a). The electrical stimulation of RMTg inhibits the spontaneous activity of VTA dopaminergic neurons (Lecca, Melis, Luchicchi, Muntoni, & Pistis, 2011b) suggesting that RMTg neurons are excited by aversive stimuli such as a foot-shock and reward-omission, whereas they are inhibited by appetitive stimuli like reward delivery (Jhou, Fields, Baxter, Saper, & Holland, 2009). CB1 receptor activation by WIN55,212 attenuates RMTg-evoked suppression of VTA DA neuron firing as well as GABA synaptic transmission on DA neurons (Lecca, Melis, Luchicchi, Muntoni, & Pistis, 2011b).

Given that CB1 receptor distribution in the RMTg largely unknown, two possible ways can be suggested in which CB1 receptors on inhibitory terminal exert their effect on VTA DA neuronal firing. First, lateral habenula (LHb), one of the major inputs to RMTg, contains a higher level of CB1 mRNA than other thalamic nuclei (Matsuda, Bonner, & Lolait, 1993). RMTg is heavily innervated by glutamatergic input from LHb (Matsumoto & Hikosaka, 2007), cannabinoids possibly influence the VTA DA neurons via LHb. In fact, electrical stimulation of LHb neurons inhibits VTA neuronal activity (Matsumoto & Hikosaka, 2007). These results indicate that activation of CB1 receptors reduces the LHb glutamatergic input to RMTg, which attenuates inhibitory control to VTA so that the neurons are disinhibited. Second, it is possible that exogenous cannabinoid bind CB1 receptors in RMTg GABAergic terminal and reduce GABA release causing

disinhibition of VTA cell firing. However, like mentioned above, no information is available on CB1 expression in the RMTg so far.

Cannabinoids can reduce both glutamate and GABA via presynaptic inhibition, which have opposite effects on VTA neuronal firing. Based on published data, disinhibition through GABAergic terminals might be more predominant than disinhibition through glutamatergic terminals. Also, endocannabinoid signaling in the VTA is required for transient increases in NAc dopamine during reward seeking (Oleson et al., 2012); 2-AG signaling in the VTA tightly controls the amount of dopamine transient in the NAc as well as reward seeking behavior. Thus, it is suggested that augmentation of VTA CB1 activation by synthetic cannabinoids positively regulate transient dopamine release by increasing VTA burst firing.

3. NAc neuronal activity

The NAc is a key neural substrate of the mesolimbic pathway that receives dense dopamine input from the VTA. Transient dopamine increases in the NAc have been found during motivated behavior such as drug seeking behavior (e.g., pressing a lever for cocaine self-administration) or responding to cues for reward availability (Carelli, 2004). Electrophysiological recordings have revealed that NAc neurons change (increase and/or decrease) firing patterns in response to

natural reward (food) as well as abused drugs (cocaine) (for review, (Carelli, 2004)). However, the effect of cannabinoid on NAc neuronal firing remains to be determined.

NAc neurons are comprised of GABAergic medium spiny neurons (MSN) (more than 90%) and fast spiking interneurons (FSI), which are characterized by a slow (< 6 spikes/s) and relatively active rate (>10 spikes/s), respectively (Pennartz, Groenewegen, & Lopes da Silva, 1994). The MSNs directly receive dopaminergic input from the VTA and glutamatergic inputs from the prefrontal cortex, hippocampus, and basolateral amaygdala (Kalivas, 2004). The FSIs, as a component of local striatal microcircuits, receive efferent signals and exert their influence on neurons outside the NAc only through their impact on the MSNs (Wiltschko, Pettibone, & berke, 2010). CB1 receptors are found in fast spiking interneuron (FSI), projecting strong inhibition on medium spiny neuron (MSN) (Winters et al., 2012) as well as in MSN (Pickel, Chan, & Kearn, 2006).

Moreover, the NAc consists of two heterogeneous sub-regions, the core and the shell, which are distinct from each other in their connections, morphology, and functions. Both the core and shell are densely innervated by VTA neurons; however, the NAc core receives glutamatergic input from the prelimbic (PL) area of mPFC, whereas the shell receives glutamatergic projection from the infralimbic (IL) are of mPFC (Sesack & Bunney, 1989). The PL-NAc core connection has

been found to be critical for drug relapse in response to drug paired cues, stress, and the drug itself (LaLumiere & Kalivas, 2008; Rogers, Ghee, & See, 2008). To understand the cannabinoid induced effects on PL-NAc core connection, neural adaptation of the NAc core was focused on throughout this dissertation.

Two types of DA receptors – D1 type receptor (D1R) and D2 type receptor (D2R) - mediate the effects on NAc neuronal activity. In vitro study showed that combination of D1R and D2R agonist enhanced the NAc core neuronal firing (Seif, et al., 2011). Also, recent study showed that D1R and D2R differently modulates excitatory glutamatergic input from PL in the NAc (Wang et al., 2012); at low frequency of cortical activity, presynaptic D1R promote glutamate release, whereas postsynpatic D2R inhibit excitatory inputs. Moreover, repeated exposures to abused drugs (cocaine) differently modulate D1R and D2R containing NAc neuronal activity in terms of membrane excitability and morphology (Kim, Park, Lee, Park, & Kim, 2011). Thus, DA regulates NAc activity via two different ways, which further underlies motivated and addiction-related behavior.

Only few studies have been done regarding neural firing patterns in the NAc in response to cannabinoid treatment. Single or repeated cannabinoid exposure could dampen the PL-NAc core glutamatergic connection (Mato, Robbe, Puente, Grandes, & Manzoni, 2005; Pistis, Porcu, Melis, Diana, & Gessa, 2001; Robbe,

Alonso, Duchamp, Bockaert, & Manzoni, 2001); these studies showed that cannabinoid reduced NAc neuronal spiking rate evoked by PL stimulation, major glutamatergic projection to the NAc core. Another in vitro study revealed that CB1 receptor activation mediates DA receptor function of increasing NAc core neuronal firing (Seif, Makriyannis, Kunos, Bonci, & Hopf, 2011) implying that CB1 activation modulates the NAc discharge in response to DA release. Moreover, regarding local field potential (LFP), changes in oscillations of large numbers of neurons, only one study showed that cannabinoid agonist, CP55,940, reduced LFP at 0.1–30 Hz in the hippocampus as well as power at 30–100 Hz in mPFC (Kucewicz, Tricklebank, Bogacz, & Jones, 2011). To the best of my knowledge, there has not been any study showing cannabinoid induced effects on NAc *in vivo* recording in freely moving animals under cannabinoid exposure.

Neuronal changes in the mesolimbic pathway after chronic cannabinoid exposure

Since cannabis derivatives are usually used for a prolonged time, this dissertation concerned with ongoing changes in the reward pathway in response to repeated exposure to cannabinoids. Following repeated administration, tolerance develops to most responses of cannabinoids including locomotor effects, antinociception, and hypothermia (Maldonado & de Fonseca, 2002). In that both synthetic cannabinoid receptor agonists and THC excite CB1 receptors

in the brain, the primary effects can involve prolonged stimulation of CB1 receptors.

Repeated exposure to cannabinoids causes down-regulation/desensitization of CB1 receptors. The cannabinoid receptor binding using [^{3}H] CP 55,940 was significantly reduced in the brain after 8 days of THC treatment including cerebellum, the limbic forebrain, and the striatum (Di Marzo et al., 2000). Moreover, WIN 55212 stimulating [^{35}S]GTPY binding decreased in most brain areas after 8 days of treatment (Di Marzo et al., 2000) implying that cannabinoid tolerance is accompanied by attenuation of G-protein activity of the CB1 receptor. This finding is also consistent with results from human marijuana users. In the post-morterm brains of chronic users of marijuana, significant reduction in brain CB1 receptors has been reported (Villares, 2007); autoradiographic binding of [^{3}H] SR141716A was decreased in the VTA and NAc of marijuana users compared to those of the control group.

In addition, chronic cannabinoid exposure alters the structure/function of neurons in the reward pathway. Kolb and colleagues found that exposure to THC increases the length of the dendrites as well as the number of dendritic branches in the NAc and PFC (Kolb, Gorny, Limebeer, & Parker, 2006). Interestingly, these changes are restricted to NAc and mPFC, which are highly innervated by the VTA. Moreover, after repeated THC treatment, dopamine metabolism measured

12

by the ratio of DOPAC was decreased in the PFC but not in the NAc (Verrico, Jentsch, & Roth, 2003). Also, there are changes in the amount of endogenous cannabinoids under chronic exogenous cannabinoid treatment. Both 2-AG and AEA significantly decreased in the dorsal striatum, whereas only AEA showed an increase in the limbic forebrain, which includes the NAc followed prolonged exposure to cannabinoids.

Thus, under prolonged exposure to cannabinoids, neuronal adaptations take place such as CB1 receptor desensitization and down regulation of endogenous cannabinoids. There exists regional difference in the rate and degree of adaptive response to chronic cannabinoid exposure (Martin, Sim-Selley, & Selley, 2004). Moreover, neuronal changes in the reward circuit does not necessarily mean alteration in function; VTA neurons continued to show enhanced firing rates following repeated cannabinoid treatment same as the initial one (Cheer, Marsden, Kendall, & Mason, 2000a; Wu & French, 2000).

To assess the acute and chronic effect of cannabinoid on NAc core signaling change, I have analyzed NAc neuronal signaling on initial and repeated exposure (seven daily injections) of a cannabinoid receptor agonist, CP55,940 (0.2 mg/kg or 0.4 mg/kg) in experiment 1. To examine dopamine dynamic under cannabinoid exposure, the electrically evoked dopamine overflow was collected using fast scan cyclic voltammetry (FSCV) in experiment 2. Using kinetic analysis,

dopamine release and reuptake were assessed immediately following one or seven daily injections of CP55,940 (0.2 mg/kg, i.p.) or a vehicle.

Experiment 1: Neuronal firing patterns of Nucleus accumbens core in response to acute and chronic exposure to CP55,940

In the experiment 1, the effects of cannabinoids on NAc neuronal firing patterns and neuronal oscillation were investigated. Since cannabis derivatives are usually used for a prolonged time, this study also investigated alterations in the NAc in response to repeated exposure to cannabinoids. To this end, the recording on neuronal discharge and LFPs in the NAc after single or repeated (7 days) cannabinoid treatment was performed with in vivo electrophysiology. This experiment focused on changes in neuronal discharge in the NAc after single or repeated cannabinoid treatment to discover the influence of cannabinoids on NAc activity Based on previous data that have shown the cannabinoid-induced suppression on glutamatergic synapse of the NAc, I hypothesized that NAc neuronal activity would be reduced after cannabinoid exposures. Moreover, in that DA receptor agonist reduced striatal theta and beta oscillation (Berke, Okatan, Skurski, & Eichenbaum, 2004), cannabinoid exposure was expected to reduce slow LFP rhythms.

Experimental Procedures

1.Subjects

Male Sprague–Dawley rats (n=29) weighing 250-350 g (Harlan, Indianapolis, IN,

USA) were housed individually and allowed at least a week of habituation before surgery. Rat housing maintained a 12:12 h light–dark cycle (lights were turned on at 9:00 am) with unrestricted water and food *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University Bloomington, IN, USA.

2. Drug Administration

Two doses (either 0.2 mg/kg or 0.4mg/kg intraperitoneal [i.p.]) of CP55,940 (Tocris, Bristol, UK) or the vehicle (10% ethanol, 10% cremophor, and 80% saline) were used for the experiment. The dose of 0.2mg/kg CP55,940 (CP0.2) was chosen because it induced rewarding effect measured by conditioned place preference (Braida, Pozzi, Cavallini, & Sala, 2001a). A higher dose of 0.4mg/kg CP55,940 (CP0.4) was also tested since the effect of chronic treatment of 0.4mg/kg includes CB1 receptor reduction in the brain (Rubino, Viganò, Massi, & Parolaro, 2000) as well as increase in cocaine self-administration (Higuera-Matas et al., 2010).

3. Electrode Construction and Surgical Procedures

Electrode bundles were constructed of eight 50 um Formvar-insulated stainless steel wires (California Fine Wire, Grover Beach, CA, USA) and a 50 um uninsulated stainless steel ground wire soldered to a 10-pin connector. On the day of surgery, the electrode bundles were chronically implanted in the NAc core

16

unilaterally; rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). One hole was drilled over NAc (+1.3 anterior and ±1.3 lateral) (Paxinos and Franklin, 2001), and the underlying dura mater was carefully removed. Three additional holes were drilled for stainless steel crews. The electrode bundle was lowered (6.5–7.5 mm ventral) into the holes, and dental cement was used to cover the surgical sites. Rats were allowed at least a week for recovery from surgery before experimental sessions began.

4. Electrophysiology

After at least 7 days of recovery, the rats were placed into an open-field recording chamber (25x18 cm with outwardly angled walls 17 cm high) for two days for at least 30 min daily for habituation to the chamber. On the following day, the rats received a saline injection intended for the habituation of the i.p. injection. On the recording day (Day 1), each rat started receiving a daily i.p. injection of 0.2mg/kg or 0.4mg/kg of CP55,940 or the corresponding vehicle for 7 days. Electrophysiological data were collected on Day 1 and 7 of either CP55,940 (CP0.2 or CP0.4) or the vehicle injection for acute and chronic treatment, respectively (Fig 2).
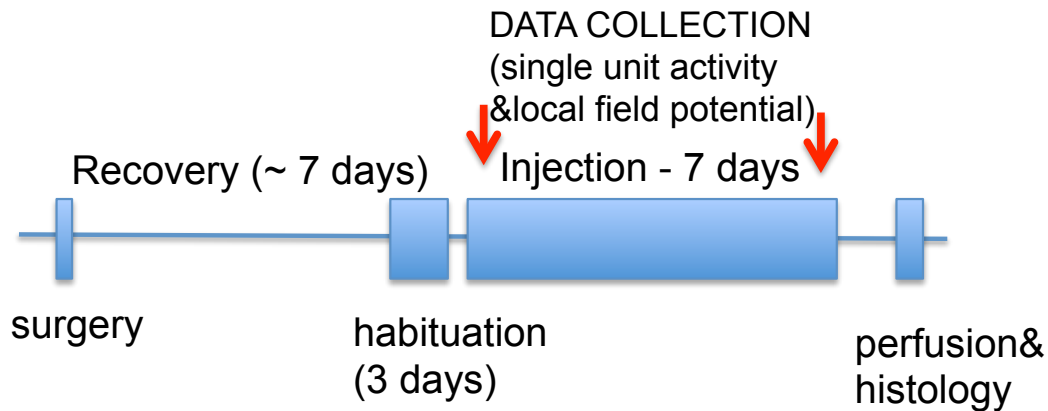
Figure 2. Timeline of experimental procedure. Electrophysiological signals collected on the first and seventh day of the treatment schedule (red arrow) following recovery from surgery.

The recording session lasted for 90 min and was divided into two successive periods: baseline (30 min), which involved no treatment, and post-injection (60 min), which began after an injection of the vehicle or CP55,940. Open field behavior was recorded for further analysis of locomotive behavior. At the beginning of recording, rats were placed into the recording chamber and the electrode assembly was connected to a lightweight flexible wire harness. The harness was connected to a commuter, which permitted unrestricted movement. Extracellular spike activity was routed through multiple-channel preamplifiers with 154Hz–8.8 kHz band pass filters (Plexon, Dallas, Texas USA). The signals were digitized at 40 Hz and registered by a multichannel acquisition processor system (Plexon). To be classified as single unit activity, waveforms displayed signal-to-noise ratios of at least 2.5:1 and exhibited a consistent shape and amplitude.

Autocorrelation was used to ensure the presence of the absolute refractory period. Putative medium spiny neuron (MSN) were selected that showed less than 10 spikes/s. LFPs were routed through preamplifiers with 1,000× gain and 0.7–170 Hz filters. At the end of the experiment, each rat was perfused for subsequent histological verification (Fig 3). Briefly, animals were deeply anesthetized and an electrical pulse (30 µA for 5 seconds) was passed through each microwire. Animals were perfused with saline followed by 10% potassium ferrocyanide in 10% formalin. Brains were removed and protected in 30% sucrose in 10% formalin. The brains were frozen and coronal slices (50 µM thick) were mounted on gelatin subbed slides to confirm the location.
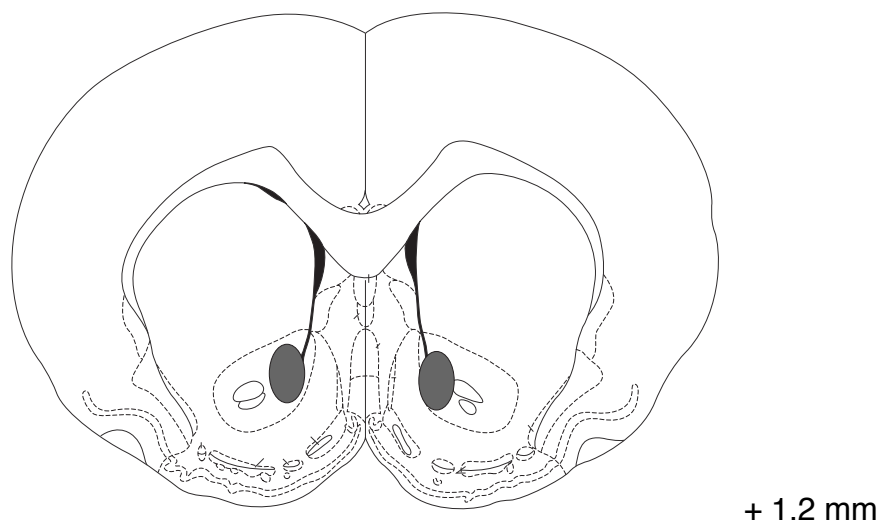

+ 1.2 mm

Figure 3. Placement of microwire electrode bundles to the NAc core (A/P +1.2 mm). Coronal section of rat brain that shows the location of histologically verified electrode bundle placements in the NAc core (modified from Paxinos and Franklin, 2001). Shaded areas (NAc) cover the places where the electrode tips were located and number indicates distance (in mm) anterior to bregma.

5. Data Analysis

Electrophysiological data were analyzed using NeuroExplorer version 4.0 (Nex Technologies, USA), MATLAB (The MathWorks, Inc.,USA) and GraphPad Prism 6 (GraphPad Software, USA). A mean baseline and post-injection firing rate were obtained during a period of 30 min before injection and 50 min after injection, respectively. The period of 10 min immediately following injection was omitted from analysis to avoid injection-related effects. The mean baseline was compared using one-way ANOVA with Bonferroni multiple comparison between three groups. The changes in firing rates of the post-injection period compared to the baseline were analyzed using a paired t- test. To examine the distribution of responses, units that changed (increase or decrease) in firing rate >30% were considered to show the significant response compared to the baseline (Ball, Wellman, Miller, & Rebec, 2010); units showing consistent response - either >30% above baseline (increase) or below baseline (decrease) - at least six consecutive bins (bin = 5 min) were classified to be excited or inhibited, respectively. Chi-square tests were used to compare the distribution of three response types—namely, inhibited, excited, and nonresponsive—among the three groups and within the same treatment group in acute and chronic treatment.

Correlated neuronal activities were examined by building cross-correlation histograms (CCHs) based on 1ms bins and ± 1 s time zero bin (NeuroExplorer). If two neurons tend to fire at the same time more than randomly, the time lag

between two neurons are zero which results in a peak at zero bin in CCHs. Peaks that exceed the 95% confidence limit in the CCHs were considered correlated whereas peaks below this limit were considered non-correlated. Fig 4 shows represented CCH matrices of non-correlated and correlated pairs (dashed line means 95% confidence limit).
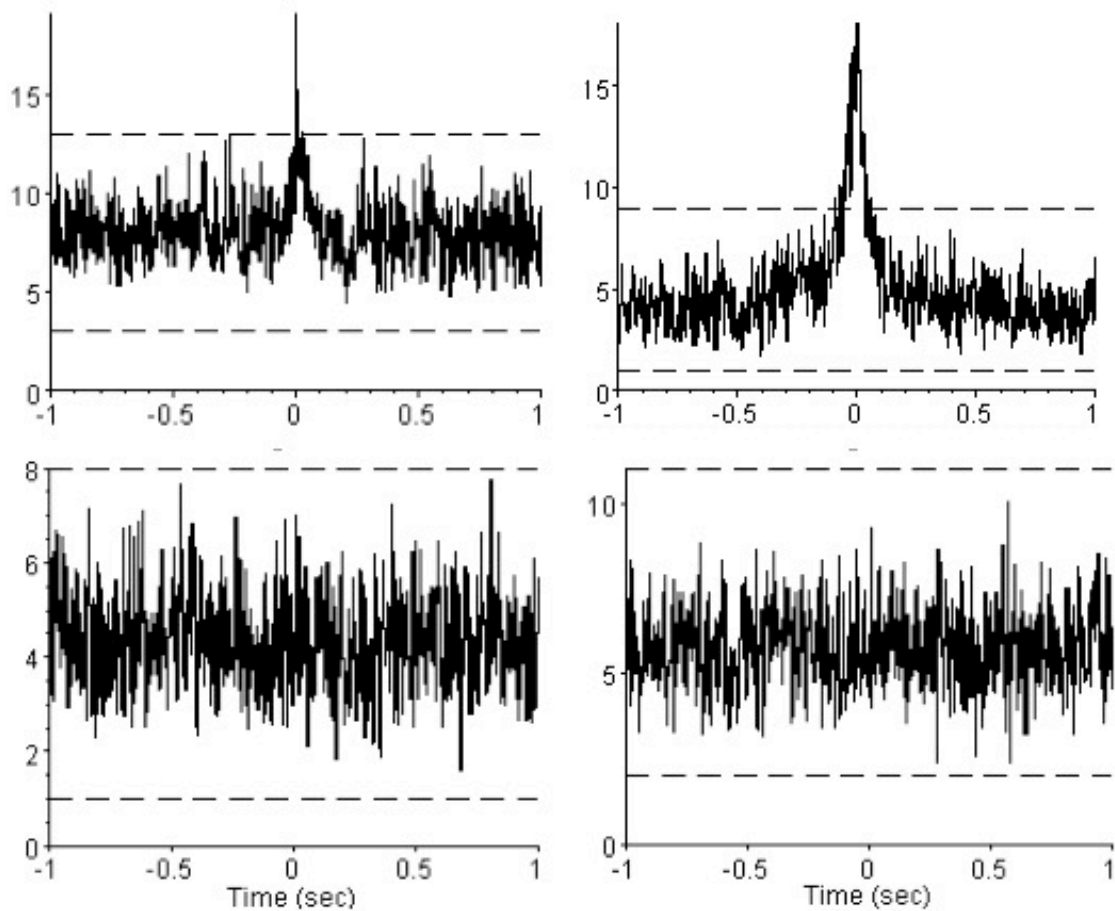
Figure 4. Representative CCHs. Y axis refers to counts/bin (bin=0.001 sec). The CCHs in the upper panel represent examples of 'correlated' pairs whereas the CCHs in the lower panel indicate examples of 'uncorrelated' pairs.

21

Burst firing was analyzed to separate fast firing activity from regular pace making firing. The specific interval method was used with the following index: the maximum interval to start burst=80ms, maximum interval to end burst=160ms, minimum interval between bursts=45ms, minimum duration of burst=20ms, and minimum number of spikes in a burst=3 based from the previous established definition (Fig 5, (Gulley & Stanis, 2010; Sun & Rebec, 2006))
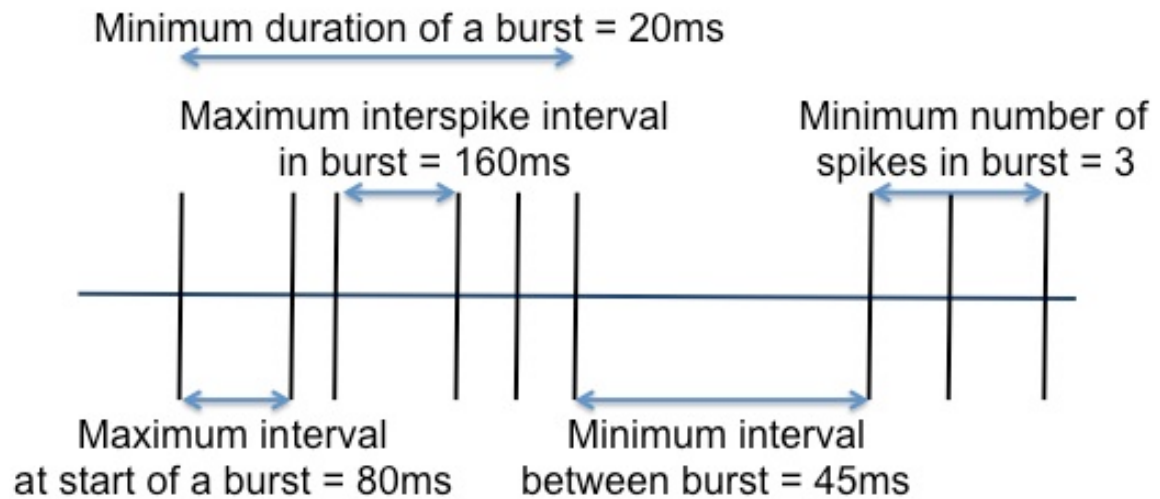
Figure 5. Diagram of burst firing analysis with specific interval methods. The parameters were adapted from the previous established literature (Gulley & Stanis, 2010; Sun & Rebec, 2006)

For LFPs, power spectral density, which was normalized and represented as a percentage of total spectral power, was compared before and after the injection. Power spectral densities (PSD) were constructed in NeuroExplorer with following index: maximum frequency = 50Hz, Number of frequency value = 256, normalized by percentage of total power spectral density (PSD), smooth with

22

Gaussian filter) (Hong et al., 2012). PSDs were averaged across three groups on day 1 and day 7 and compared. Spectrogram generated in NeuroExplorer using low PSD across the time.

Quantifying locomotion, a trained observer viewed DVDs of experimental sessions using JWathcer (University of California Los Angeles, USA). Locomotion was defined the time spent when animals step forward with all four paws moving. To assess the level of cannabinoid-induced locomotion, the time of locomotive activity after vehicle or CP55,940 injection was normalized to that of a baseline. The locomotive behavior was compared using one-way ANOVA with Bonferroni's multiple comparison between three groups

RESULTS

1. Mean Firing Rate Changes after CP55,940 treatment

For the recording, the rats were placed in the open arena, and the baseline neuronal firing rate was acquired during the first 30 min prior to the injection with the vehicle or either dosage of CP55,950. The mean baseline firing rates (±SEM) were 2.51 (±0.50), 2.95 (±0.63), and 2.37 (±0.51) per second for the vehicle, 0.2 mg/kg of CP55,940 (CP0.2), and 0.4 mg/kg of CP55,940 (CP0.4) group, respectively (Fig. 6; the number of neurons : vehicle = 32, CP0.2 = 28, CP0.4 = 33). There was no significant difference across the three groups in the baseline firing rate tested by one-way ANOVA. However, after cannabinoid injection, firing rates were significantly reduced to 1.04 (±0.31) and 1.29 (±0.42) for CP0.2 and CP0.4, respectively ($p < 0.01$ and $p < 0.001$, paired t test), whereas the vehicle injection did not show any significant change in firing rates.
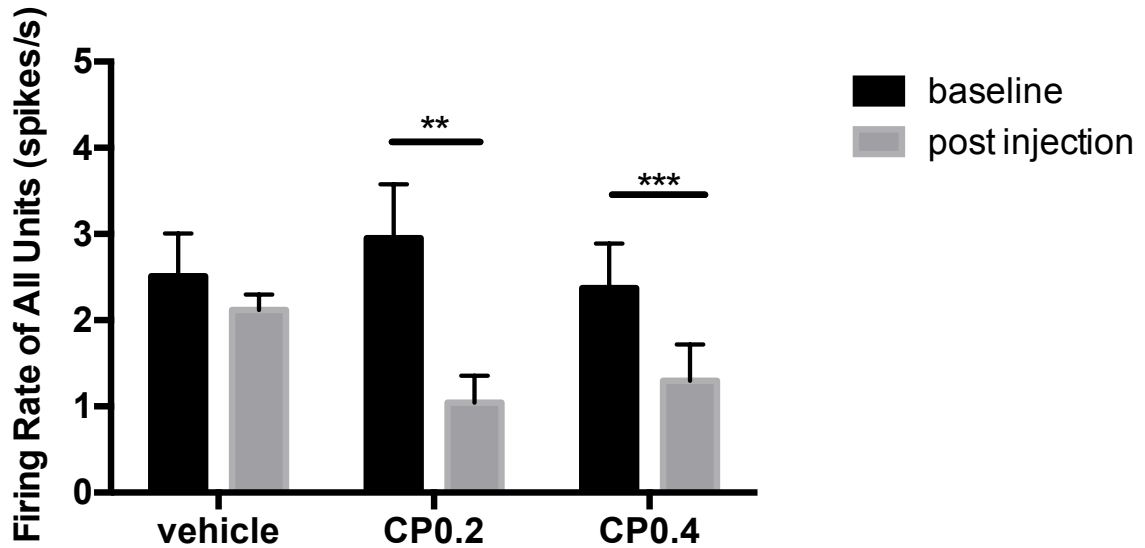
Figure 6. Effect of acute vehicle or CP55,940 treatment. Mean firing rate with SEM of all units on day1. Baseline (30min) and post-injection (50min) firing rates were compared using paired t-test, **p<0.01, ***p<0.001

Following chronic treatment (Fig 7), the baseline firing rates were not significantly different across the three groups (One-way ANOVA, the number of neurons: vehicle = 28, CP0.2 = 29, CP0.4 = 34). However, CP0.2 and CP0.4 treatment still showed significant reduction in the firing rate similar to acute treatment; the mean baseline firing rate was 2.97 (±0.68), 3.39 (±0.83), and 3.33 (±0.71) for the vehicle, CP0.2, and CP0.4 groups, respectively. After injection, CP0.2 and CP0.4 significantly decreased the mean firing rate to 1.74 (±0.66) and 1.79 (±0.50), respectively ($p < 0.001$ and $p < 0.01$, paired t test), whereas the vehicle injection again did not show any significant change in firing rates.
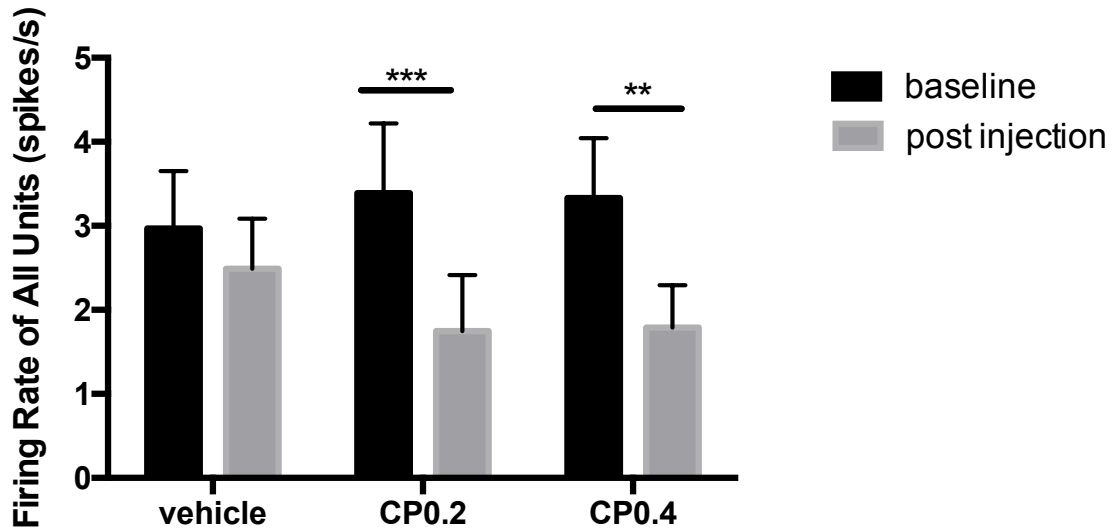
Figure 7. Effect of vehicle or CP55,940 chronic treatment. Mean firing rate with SEM of all units on day 7. Baseline and post-injection firing rates were compared using paired t test, ** p <0.01, *** p <0.001

2. Distributions of Inhibited, Excited, or Non-responsive Units

Units showing either consistent increases or decreases in firing rate >30% from the baseline for at least six consecutive five minute bins (30 min) were classified as either excited or inhibited, respectively. Following to acute treatment, most neurons (86% and 76% of the total units for CP0.2 and CP0.4, respectively) showed significantly inhibited responses, whereas 60% of neurons were non-responsive to the acute vehicle treatment (Fig. 8). Chi square test confirmed the significant differences in neuronal response (χ^2 = 69.31, df =4, p <.0001). The acute vehicle group showed significant difference compared to CP0.2 and CP0.4 (CP0.2: χ^2 = 56.72, df =2, p < 0.0001; CP0.4 χ^2 = 37.04, p < 0.0001). The responses of the two cannabinoid-treatment groups did not show any significant
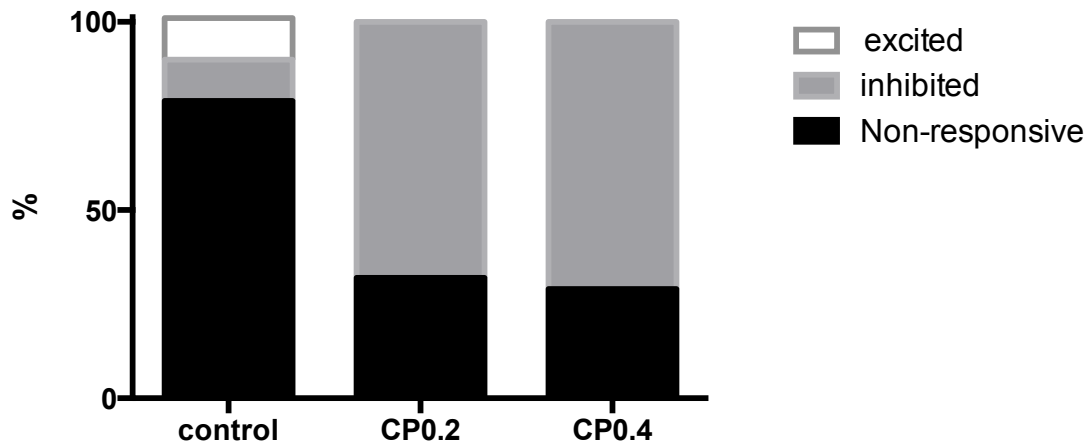
differences in the distribution of responsive units, which implies that the two different doses caused similar effects on neuronal response.
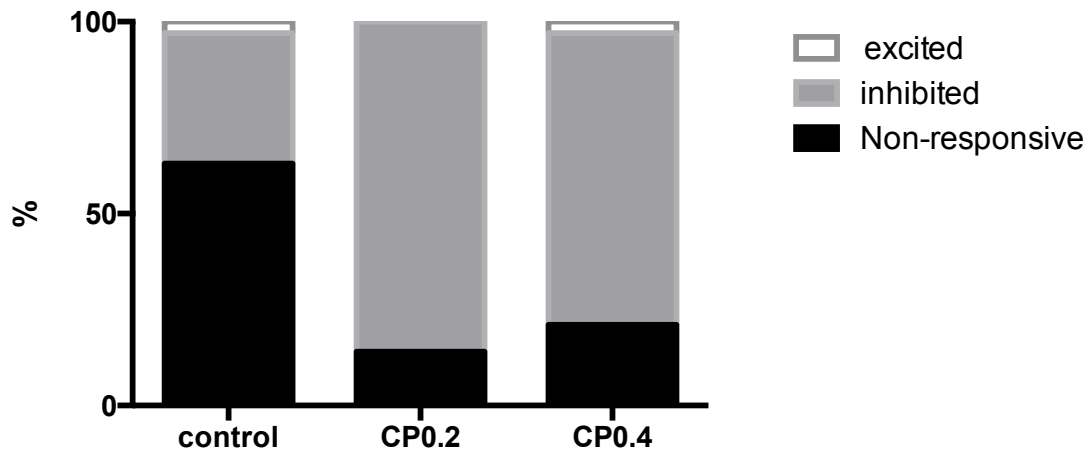
Figure 8. Distribution of excited, inhibited and non-responsive neurons following acute vehicle or CP55,940 treatment Chi statistic shows there are significant differences between control vs. CP0.2 and control vs. CP0.4 ($p<.0001$). No significant difference in unit distribution between CP0.2 and CP0.4

Following the seventh injection of the vehicle or either CP0.2 or CP0.4 (Fig. 9), the responses of most neurons shows similar pattern compared to acute treatment; the majority of neurons in CP0.2 and CP0.4 showed inhibited response (68% and 71%, respectively), whereas 79% of neurons in the vehicle treated groups showed non-responsive. Also, chronic treatment of cannabinoid still caused different neuronal response compared to the vehicle treatment ($\chi^2 = 72.02$, $p < 0.0001$ for CP0.2; $\chi^2 = 78.05$, $p < 0.0001$ for CP0.4). No significant difference in distribution of responses between two cannabinoid groups on Day 7.

Within the same treatment group, the prolonged treatment of the vehicle or CP0.2 changes in distribution of neuronal response compared Day 1 with Day 7 (χ^2 = 18.13, p = 0.001 for the vehicle; χ^2 = 9.147, p < 0.01 for CP0.2). Treatment of CP0.4 showed the same response.

Figure 9. Distribution of excited, inhibited and non-responsive neurons following chronic vehicle or CP55,940 treatment. Chi statistic shows there are significant differences between control vs. CP0.2 and control vs. CP0.4 (p<.0001). No significant difference in unit distribution between CP0.2 and CP0.4.

3. Response of NAc neurons across time after injection

In response to the acute treatment, the mean firing rate of all units significantly decreased compared to the baseline by 46% and 59% for CP0.2 and CP0.4, respectively, after 16–20 min post-injection (Fig. 10). After 20 min post-injection, the mean firing rate was reduced by 80% and 70% for CP0.2 and CP0.4, and the changes in magnitude remained significantly decreased compared to the vehicle

treatment. One-way ANOVA with Bonferroni multiple comparisons test revealed that % of baseline of three groups were significantly different after 16 min of injection and the differences were from CP0.2 vs. vehicle and CP0.4 vs. vehicle. Difference between CP0.2 and CP0.4 were not significant at any time bin.

Day 1
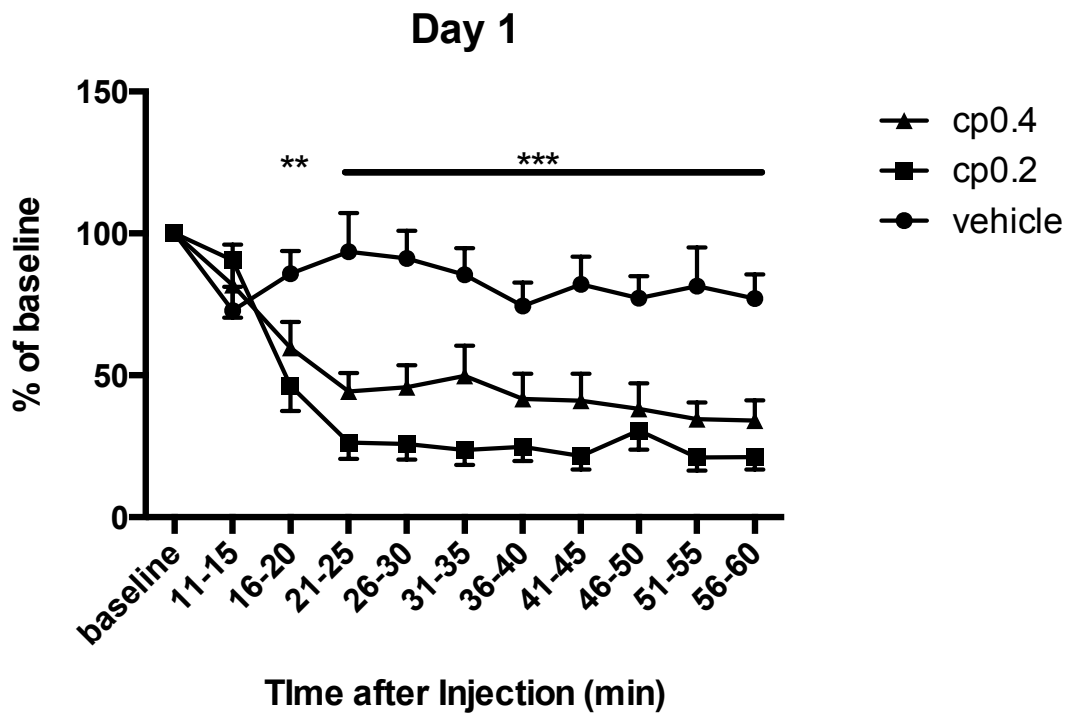
Figure 10. Magnitude of response for mean firing rate after acute vehicle or CP55,940 treatment. Curves represent mean (+SEM) firing rate normalized to pre-injection baseline activity for each 5-min bin beginning 10 min after CP55,940 (or vehicle) injection on Day1. Averaged response of all recorded units showed significant reduction after CP55,940 compared to vehicle (one-way ANOVA). Between CP0.2 vs. CP0.4 there were not significant differences. (** $p < .01$, ***$p < .001$)

Table 1. The magnitudes of firing rate changes compared to the baseline (% of baseline) across time in acute treatment. The average ± SEM

	vehicle	CP0.2	CP0.4
11-15 min	72.80 ± 8.35	90.50 ± 20.16	81.78 ± 14.25
16-20 min	72.80 ± 8.09	46.32 ± 8.89	59.69 ± 9.11
21-25 min	85.77 ± 13.52	26.34 ± 5.81	44.36 ± 6.48
26-30 min	93.62± 9.64	25.79 ± 5.43	45.86 ± 7.74
31-35 min	91.24 ± 9.37	23.71 ± 5.33	49.84 ± 10.57
36-40 min	85.39 ± 8.26	24.79 ± 5.02	41.7 ± 8.8
41-45 min	74.46 ± 9.83	21.54 ± 4.75	41.09 ± 9.48
46-50 min	82.02 ± 7.84	30.6 ± 6.85	38.23 ± 9.01
51-55 min	77.16 ± 13.60	21.04 ± 4.62	34.62 ± 5.85
56-60 min	81.48 ± 8.49	21.14 ± 4.38	34 ± 7.14

Following the chronic treatment, the patterns were same as those of the acute treatment (Fig. 11). Average single unit activity remained inhibited by about 40–50% (compared to the baseline) at 10 min after injection CP0.2 or CP0.4 (Table 2). One-way Anova with Bonferroni test (multiple comparisons) revealed that % of baseline of three groups were significantly different after 11 min of injection and the differences are from CP0.2 vs. vehicle and CP0.4 vs. vehicle.
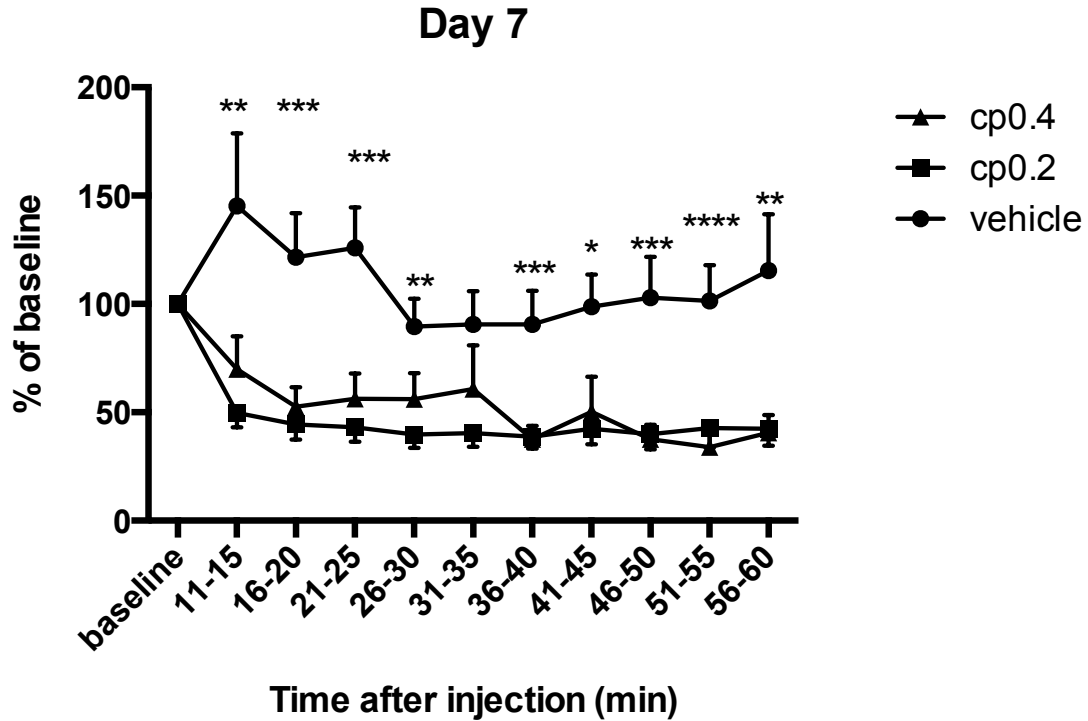
Figure 11. Magnitude of response for mean firing rate after chronic vehicle or CP55,940 treatment. Curves represent mean (+SEM) firing rate normalized to pre-injection baseline activity for each 5-min bin beginning 10 min after CP55,940 (or vehicle) injection on Day7. Averaged response of all recorded units showed significant inhibition after CP55,940 similar to Day 1. *p<.05, **p<.01, ***p<.001 , one way ANOVA with Bonferroni's multiple test.

Table 2. The magnitudes of firing rate changes compared to the baseline (% of baseline) across time in chronic treatment. The average ± SEM

	vehicle	CP0.2	CP0.4
11-15 min	145.26 ± 33.57	49.73 ± 6.66	69.93 ± 15.18
16-20 min	121.65 ± 20.33	44.44 ± 6.97	52.58 ± 8.91
21-25 min	125.93 ± 18.71	43.02 ± 6.55	56.20 ± 11.77
26-30 min	89.65 ± 12.71	39.71 ± 6.17	56.05 ± 11.96
31-35 min	90.60 ± 15.33	40.34 ± 6.19	60.80 ± 20.15
36-40 min	90.58 ± 15.50	38.75 ± 5.49	37.62 ± 6.14
41-45 min	98.74 ± 14.76	42.35 ± 7.09	50.31 ± 16.04
46-50 min	102.88 ± 18.79	39.97 ± 7.02	37.62 ± 6.60
51-55 min	101.36 ± 16.52	42.79 ± 9.02	33.99 ± 7.58
56-60 min	115.47 ± 25.89	42.41 ± 7.87	40.65 ± 8.07

To compare the response between acute and chronic treatment of CP55,940, response of each bin (5 min) on day1 was compared to the same bin on day7 using t-test. Acute treatment of 0.2mg/kg of CP55,940 significantly inhibited the firing rates more than chronic treatment (Fig 12 (a); 21-25, 31-45, 55-60 min after the injection showed significant reduction on acute treatment compared to the chronic treatment, $p < .05$). However, 0.4mg/kg of CP55,940 treatment showed consistent response on both acute and chronic treatment (Fig 12 (b)).
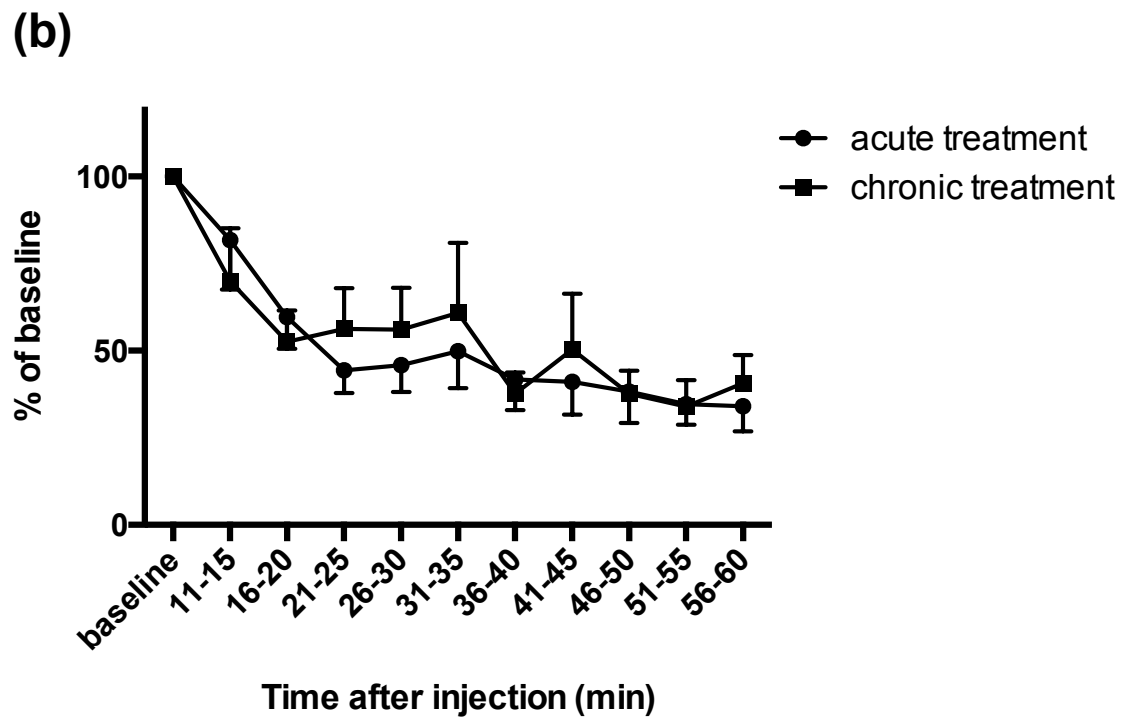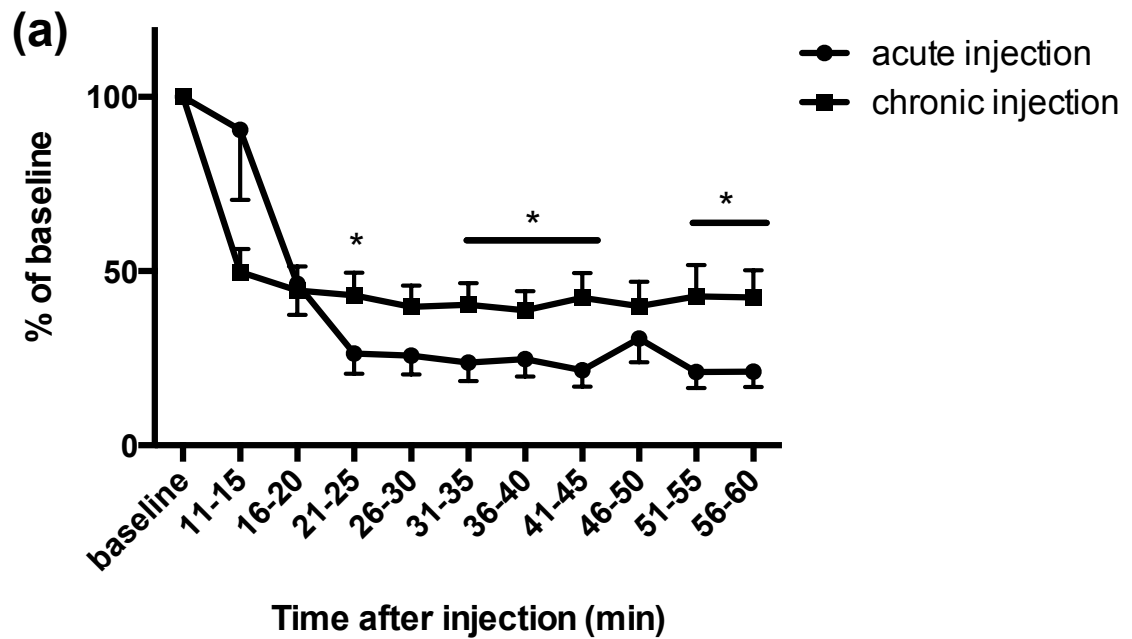
Figure 12.Magnitude of neuronal responses of CP0.2 (a) and CP0.4 (b) in response to acute or chronic treatment. Neuronal responses after acute treatment and chronic treatment were compared using t-test. (a) In CP0.2 group, acute treatment showed significant reduced neuronal firing activity compared to

chronic treatment (*p<.05). (b) CP0.4 group showed no significant difference between acute and chronic treatment.

4. Cross-correlated firing

Correlated neuronal activities were examined by building cross-correlation histogram (CCH). The CCHs were constructed for all pair-wise comparisons within each recording session. If two neurons tend to fire at the same time more than randomly (Fig 13), the time between two neurons are zero which results in a peak at zero in CCHs. Peaks that exceed the 95% confidence limit in the CCHs were considered correlated whereas peaks below this limit were considered non-correlated (two neurons fire independently).

Figure 13. Example of rasters from one pair of neurons (units). Cross-correlation highlighted in grey and occurs two neurons fire together.

Before the injections, 50%, 48%, and 49% of pairs showed correlated activities in vehicle, CP0.2, and CP0.4 group respectively (total number of pairs: vehicle = 40 pairs; CP0.2 = 46 pairs; CP0.4 = 45 pairs). However, in response to the initial injection of CP55,940, the proportion of correlated pairs were significantly decreased by 33% and 29% for CP0.2 and CP0.4 respectively (Fisher's exact

test; $p<.05$ for CP0.2 and $p<.01$ for CP0.4) whereas vehicle injection slightly changed it by 45% (Fig 14).
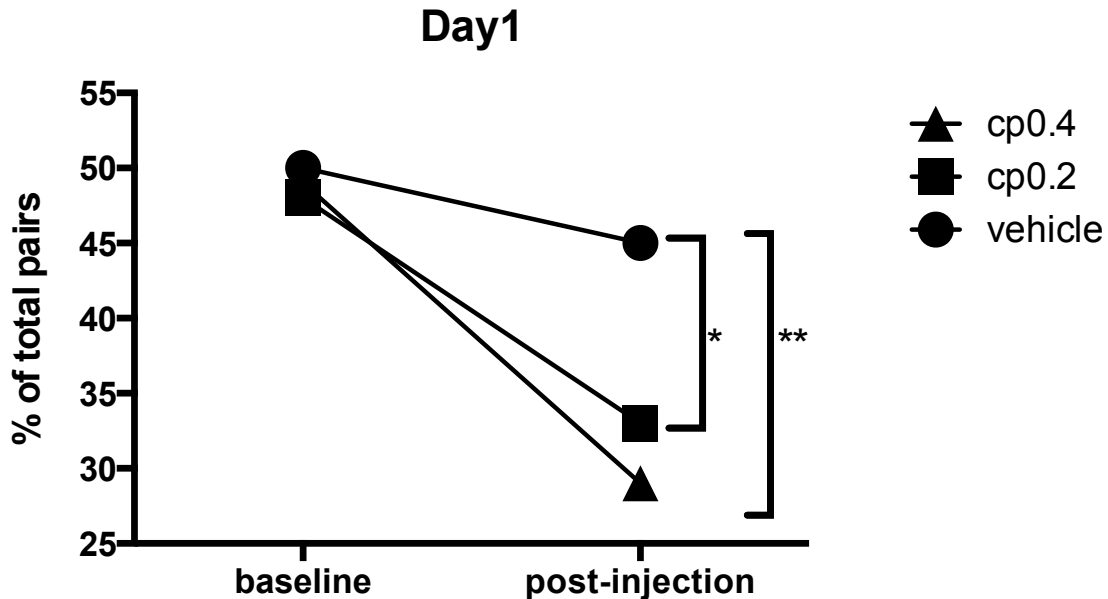
Figure 14. Change in proportion of correlated pairs following acute vehicle or CP55,940. The proportion of correlated pairs were significantly decreased after the injection of cp0.2 and cp0.4 compared to the baseline on day 1 (Fisher's exact test. *$p<0.5$ for cp0.2 and **$p<.01$ for cp0.4)

On seventh day of the injection, the patterns remain similar (Fig 15). The 44% (vehicle), 41% (CP0.2) and 43% (CP0.4) of pairs show correlated neuronal firing before the injection (total number of pairs: vehicle = 46 pairs; CP0.2 = 61 pairs; CP0.4 = 56 pairs). During the post-injection period, 50% of paired neuron in vehicle group showed correlated activity whereas only 21% (CP0.2) and 27% (CP0.4) showed correlated, which is significantly reduced compared to the baseline (Fisher's exact test; $p<0.1$ for CP0.2 and $p<.05$ for CP0.4).
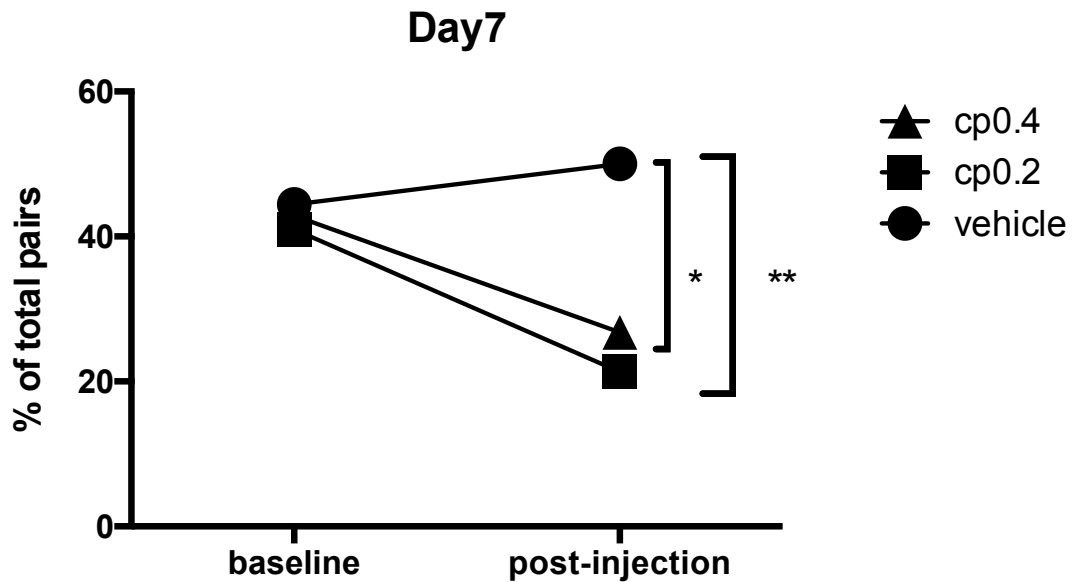
Figure 15. Change in proportion of correlated pairs following chronic vehicle or CP55,940. The proportion of correlated pairs were significantly decreased after the injection of cp0.2 and cp0.4 compared to the baseline on day 7 (Fisher's exact test; $p < 0.1$ for cp0.2 and $p < .05$ for cp0.4)

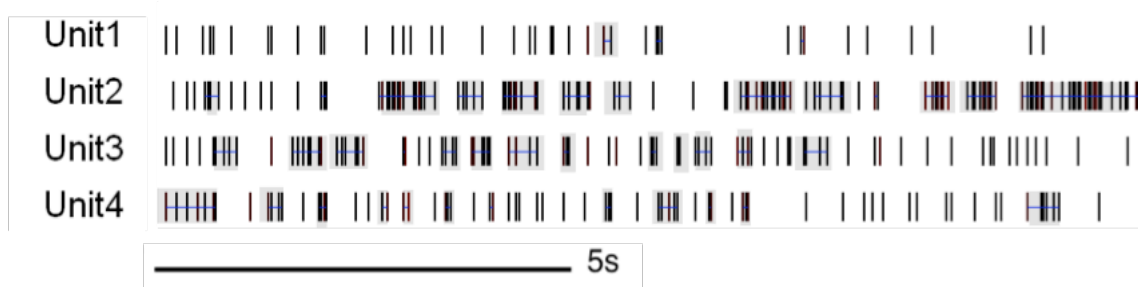## 5. Burst firing rate

Burst firing (see Fig 16 for a representative example) was measured using the specific interval algorithm on day 1 and day 7. The burst rates (bursts/min) and % of spikes in bursts were calculated.

Figure 16. Representative examples of burst firing in four different neurons. The gray shading and blue lines indicate bursts in each spike raster

The burst firing rates in baseline were not significantly different across three groups on day 1 (Fig 17; the mean burst rate (bursts/min ± SEM) was 11.85 (± 2.83) for vehicle; 15.35 (± 3.63) for CP0.2; 12.17 (± 3.25 for CP0.4). (Statistically not significant (n.s.) in one way ANOVA analysis). After the acute injection, the burst rates of CP0.2 and CP0.4 were significantly decreased to 5.22 (±1.98) and 6.40 (± 2.20), respectively whereas that of vehicle (9.31± 2.47) was not significantly changed (paired t-test; $p<0.01$ for both CP0.2 and CP0.4).
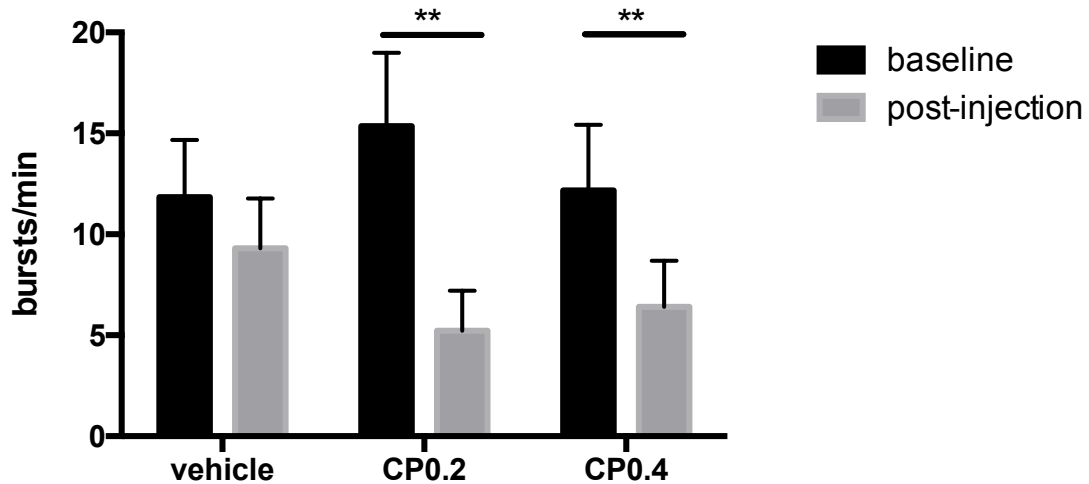
Figure 17. Burst rate per minute following acute vehicle or CP55,940. The baseline burst rate was compared with one-way ANOVA across three groups (n.s.) and paired t-test was performed to compare the change within each group. **$p<.01$

Moreover, the percentage of spikes in burst firing was compared before and after the injection. About one third of total neuronal spikes were categorized as burst firing during the baseline period and there was no significant group differences using one way ANOVA on day 1 (Fig 18); 34.89 ($\pm$ 4.55)% for vehicle, 42.33 ($\pm$ 4.55) for CP0.2 and 34.16 ($\pm$ 4.02). However, after the injection, CP0.2 and CP0.4 group showed significant reduction on day 1 whereas vehicle group did not show any significant difference (paired t-test; $p<.01$ for both CP0.2 and CP0.4 group, n.s for vehicle group; the percentage of spikes in burst = 33.57 ($\pm$ 4.27) for vehicle, 30.02 ($\pm$ 4.28) for CP0.2 and 21.54 ($\pm$ 4.03) for CP0.4.
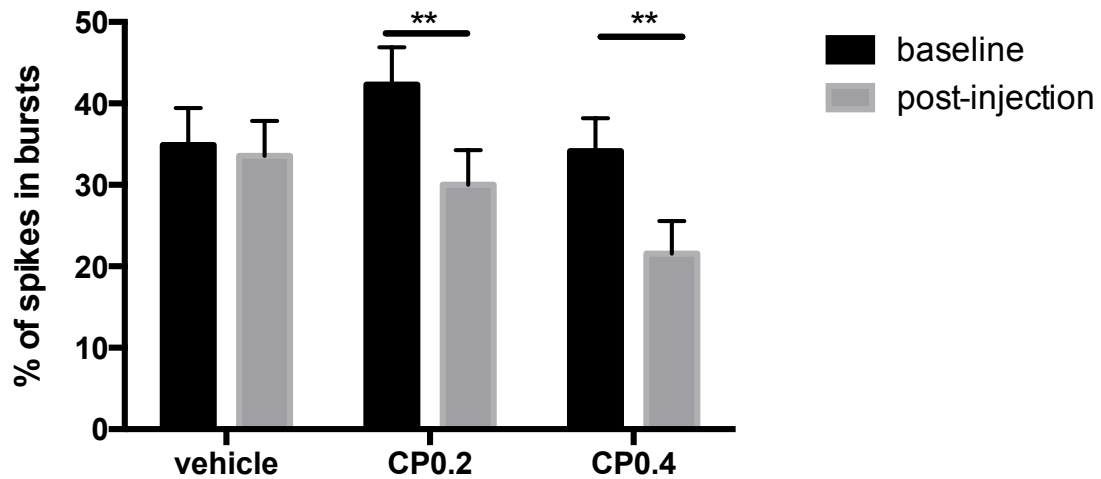
Figure 18. Percentage of spikes in burst firing following acute vehicle and CP55,940. Paired t-test was performed within each group. **$p<.01$

On seventh day (Fig 19), the baseline burst rates are not different across three groups tested by one way ANOVA (bursts/rate ± SEM; 13.84 ±3.65 for vehicle, 15.98 ±4.17 for CP0.2, 16.35 ± 3.91 for CP0.4 ; the number of neurons : vehicle = 28, CP0.2 = 29, CP0.4 = 34). Similar to the response of day 1, CP55,940 injection significantly reduced the burst rate whereas vehicle did not; the mean burst rate decreased to 7.32 (± 3.28) for CP0.2 and 9.65 (± 3.33) for CP0.4, and 11.58 (± 3.19) for vehicle. (paired t-test; $p<.01$ for both of CP0.2 and CP0.4).
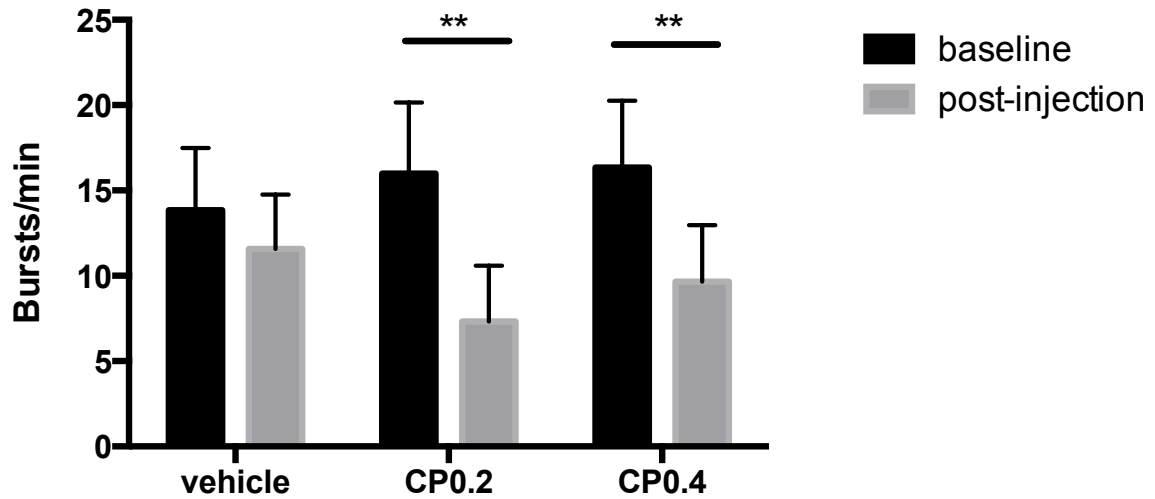
Figure 19. Burst rate per minute following chronic vehicle or CP55,940. The baseline burst rate was compared with one-way ANOVA across three groups (n.s.) and paired t-test was performed to compare the burst rates within each group. **$p<.01$

Moreover, the percentage of spikes in bursts on day 7 showed similar patterns as day 1 (Fig 20); the baselines of three groups were 36.25 (± 4.83) for vehicle group, 37.58 (± 5.22) for CP0.2 group and 37.18 (± 5.19) for CP0.4 group (statically not significant). After the injections, the percentage of spikes dropped to 19.56 (± 4.69) and 26.47 (±4.85) for CP0.2 group and CP0.4 group, respectively whereas it remained similar to the baseline for the vehicle group (37.05 ± 4.77). (paired t-test, $p<.0001$ for CP0.2 and $p<.01$ for CP0.4)
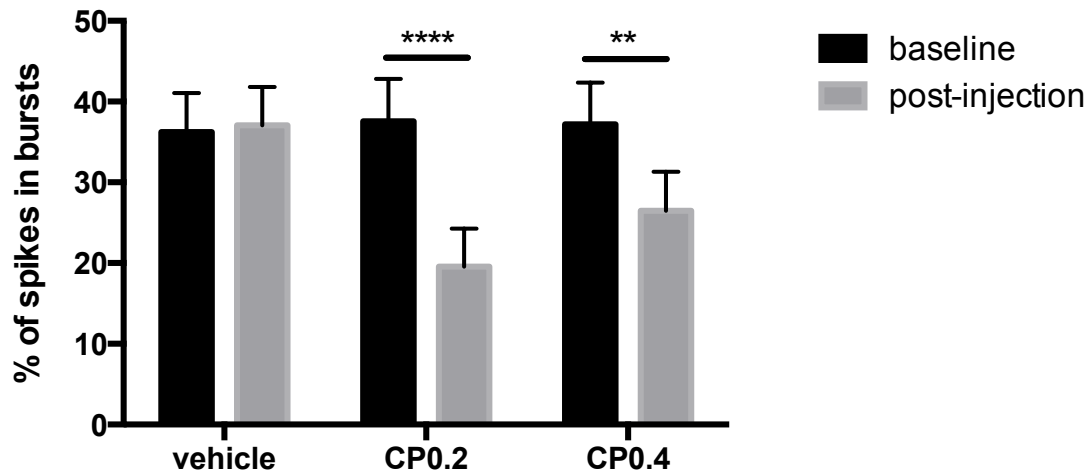
Figure 20. Percentage of spikes in burst firing following chronic vehicle or CP55,940. Paired t-test was performed within each group. ***p*<.01, *****p*<.001

6. Local field potentials

Power spectral densities (PSD) were normalized by percentage of total PSD and smoothly with Gaussian filter (filter width =3) (NeuroExplorer). The percentage of total PSD was averaged on the subjects in the same group and the % of PSD of the baseline and post-injection were compared using independent t-test with Bonferroni correction.

In response to vehicle treatment, the averaged % PSD of baseline was not significantly different to that of post-injection period (Fig 21 (a)(b)). For both CP0.2 and CP0.4 group, the power on theta spectrum (4-8 Hz) increased after the acute injection compared to baseline (Fig 21 (c),(e)). Following chronic treatment, dominance in theta band disappeared in CP0.4 group but CP0.2

41

showed higher PSD at 5-5.8 Hz compared to baseline (Fig 21 (d), (f)). In addition to the theta band, PSD at 1.3-2Hz showed higher densities after acute CP0.4 treatment (Fig 21 (e)). Spectrogram showed similar density in baseline and post-injection after chronic treatment of CP0.4 (Fig 21 (f)).
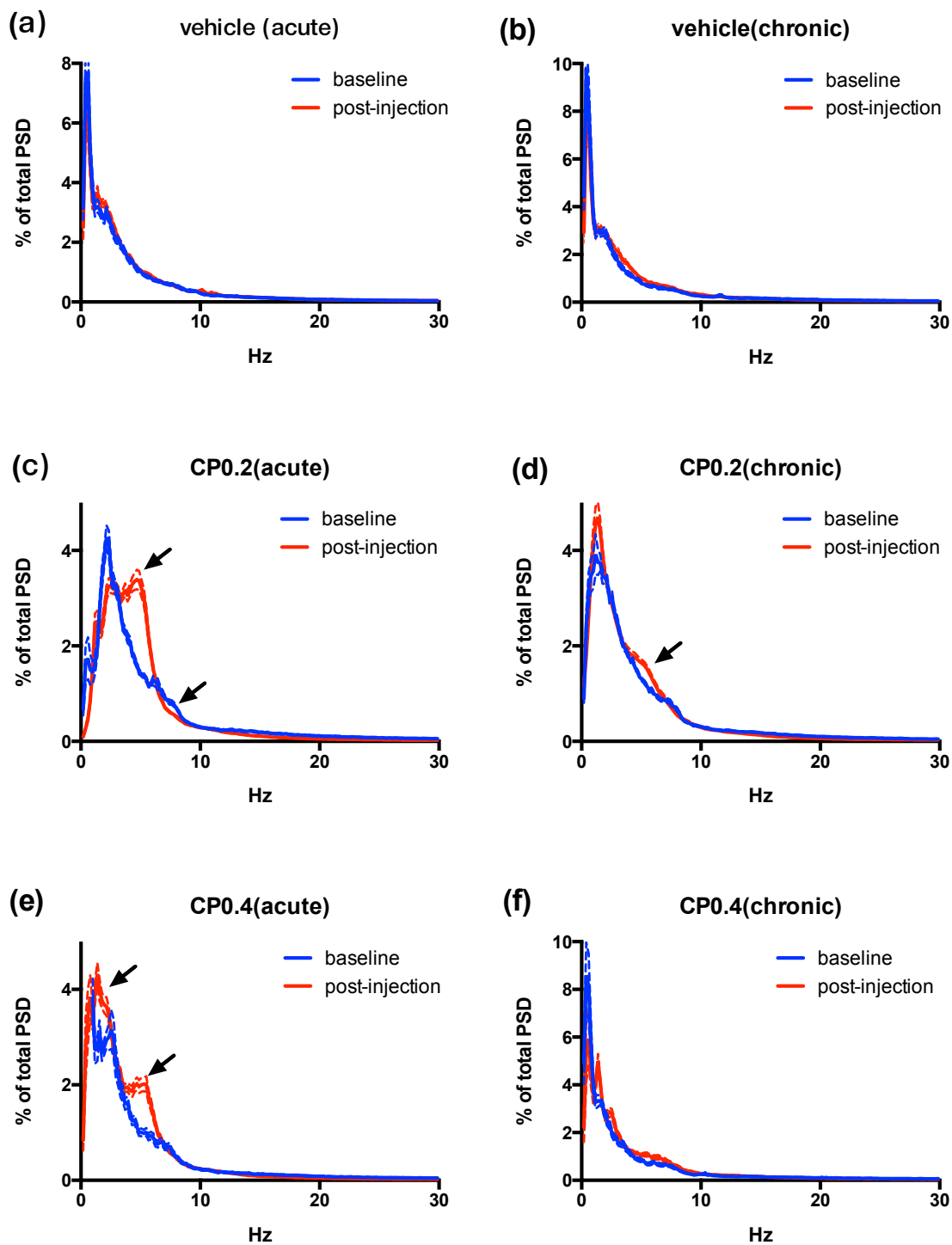
Figure 21. Mean Power Spectral Density (PSD) plots of vehicle (a)(b), CP0.2 (c)(d) and CP0.4 (e)(f) group. Mean percentage of total PSD was calculated (solid line = mean, dotted line = SEM) following acute or chronic treatment in

each condition. Black arrows mark the frequency peaks that showed significant group differences (independent t-test with Bonferroni correction).
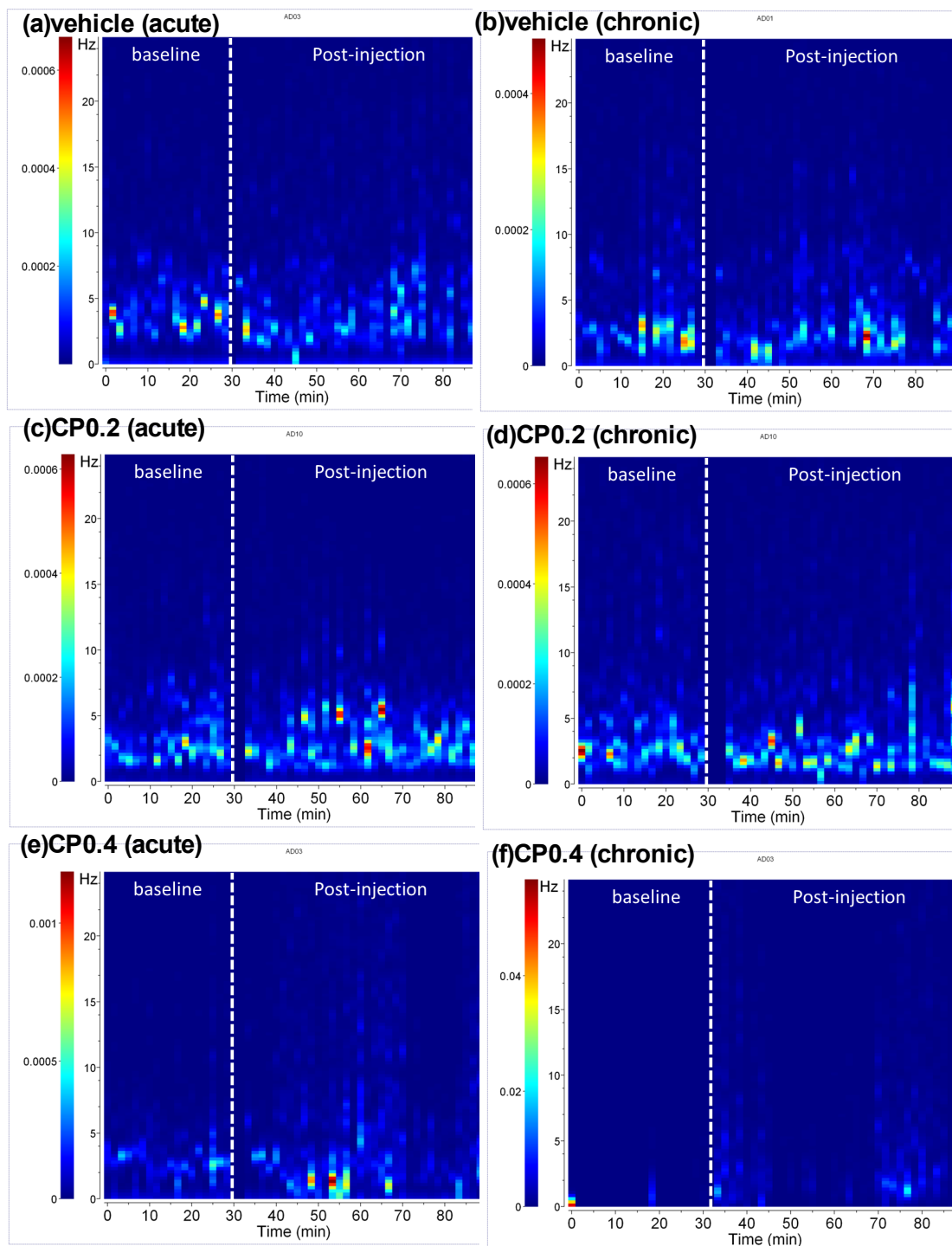
Figure 22. Examples of spectrogram after acute and chronic treatment with vehicle (a)(b), CP0.2 (c)(d), and CP0.4 (e)(f); Left panels indicate acute treatment ((a)(c)(e)) and right panels indicate chronic treatment ((b)(d)(f)). Time-frequency plots show changes in power spectral density (PSD) in vehicle, CP0.2

and CP0.4 groups. Animals received injection of vehicle or CP55,940 at 30 min (white dotted line). The theta rhythms showed greater spectral power density after acute cannabinoid treatment.

7. Locomotion

The doses used in this study significantly suppressed locomotor activity after acute treatment; Acute injection of P55,940, both 0.2mg/kg and 0.4mg/kg, significantly reduced locomotor activity compared to vehicle ($p<.01$, one way ANOVA, Fig 23 (a)). However, this cannabinoid-induced decrease in locomotion was no longer present after chronic treatment (Fig 23 (b)). The levels of locomotion of CP0.2 and CP0.4 were not significantly different from chronic vehicle.
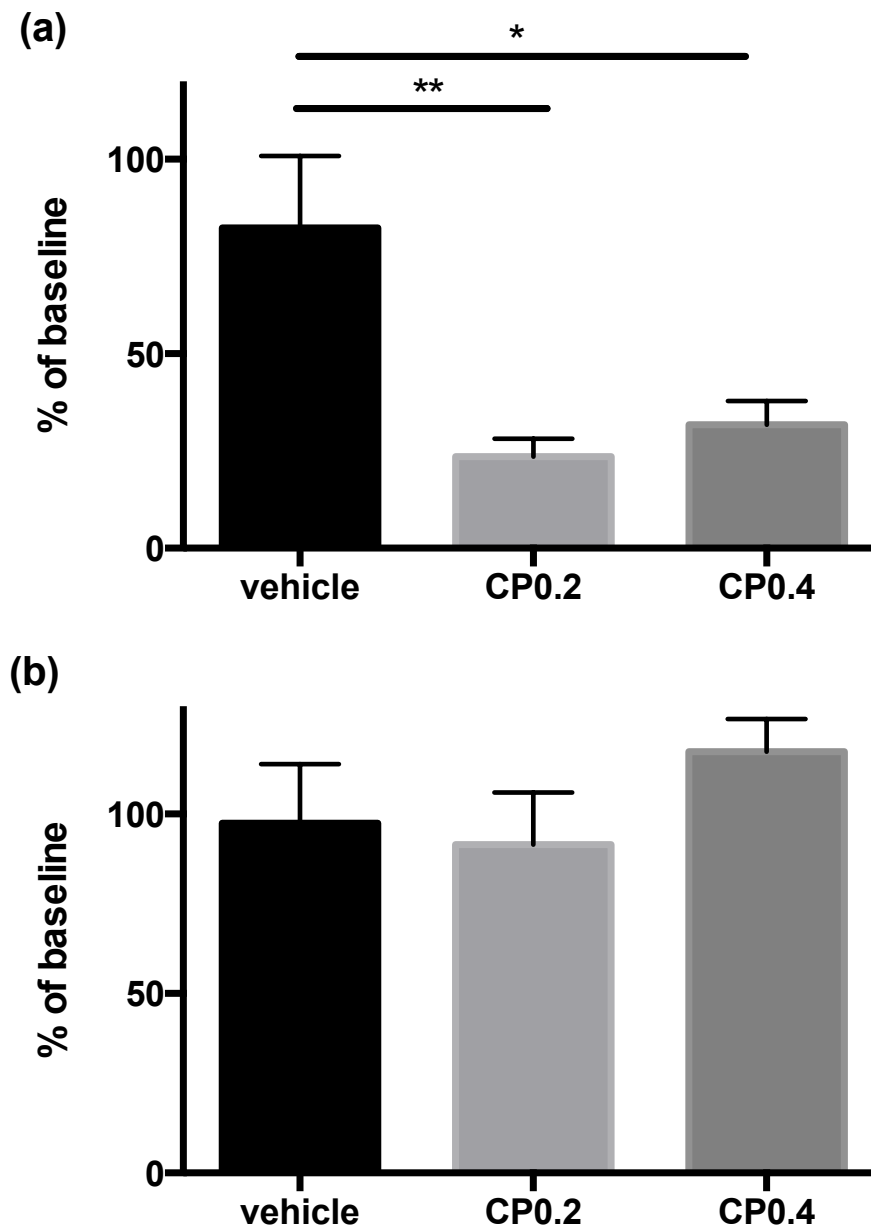
Figure 23. Locomotion after acute treatment (a) and chronic treatment (b).
(a) Both CP0.2 and CP0.4 showed significantly reduced locomotor activity after acute injection (one way ANOVA with Bonferroni's multiple comparisons test (*p<.05, **p<.01). (b) No significant differences across three groups (one way ANOVA).

Discussion

The experiment 1 showed that CB1/2 receptor activation by CP55,940 inhibited NAc neuron activity supporting the hypothesis. This inhibition in neuronal activity appeared about 10 min after the cannabinoid injection, and the magnitude of inhibition was about 40-50% of the baseline. This inhibition in firing rates was also found after seven days of repeated CP55,940 treatment; the chronic treatment of 0.2 mg/kg CP55,940 slightly attenuated the inhibition, but NAc neurons still showed significant reduction in firing rates compared to vehicle treatment. Moreover, the chronic 0.4 mg of CP55,940 treatment maintained the consistency of inhibited response on the initial treatment.

The results of the present experiment also indicate that cannabinoid treatment reduced bursts and correlated activity between NAc neurons. Burst spikes increase the chance of triggering post-synaptic transmission (Izhikevich, Desai, & Walcott, 2003). Moreover, correlative neuronal activity implies that they are in the same receptive field-overlap of presynaptic neurons (Salinas & Sejnowski, 2001). Thus, both changes in bursts and correlative activity may explain alteration in functional communication between adjacent neurons. The reduction in bursts as well as correlative neural activity observed in this study may imply decreased functional connection, as well as disrupt information processing within NAc. However, in the present study, the reduction in correlated and burst firing is also

likely due to the decrease in firing rate, relative to the baseline. Since the probability of correlated firing increases in spike trains with higher firing rates (B. R. Miller, Walker, Shah, Barton, & Rebec, 2008), decrease in correlated neuronal activity may possibly due to the reduction in neuronal firing rates.

The possible underlying mechanism of reduction in firing rates as well as the correlated and bursts activity of NAc might be due to the cannabinoid-induced inhibitory input to the NAc core. In other words, the overall output of cannabinoid-induced presynaptic regulation on the NAc core neurons potentially lowered neuronal excitability. For this, the inhibition of excitatory input (i.e., glutamate release) was presumably involved. The anatomical evidence that CB1 receptors were mostly found in the excitatory presynaptic dendrite in the NAc (Pickel, Chan, Kash, Rodríguez, & Mackie, 2004), instead of the inhibitory presynaptic synapses, could support these results. Thus, cannabinoids influence neuronal activities mainly by reducing excitatory input to the NAc, which is consistent with its anatomical distribution.

Medium spiny neurons in the NAc are mostly GABAergic neurons and they provide an inhibitory projection to the VTA (Kalivas, Churchill, & Klitenick, 1993).. The reduction in inhibitory NAc input to the VTA might play a role in increasing VTA neuronal excitability. The present finding of steady NAc firing inhibition under chronic cannabinoid treatment also supports previous studies (Cheer,

Marsden, Kendall, & Mason, 2000a; Wu & French, 2000) that showed consistently enhanced VTA neuronal firing after repeated cannabinoid administration. Taken together, it can be expected that persistent NAc core neuronal signaling can be involved in the development of tolerance in the mesolimbic pathway.

Interestingly, inhibited NAc neuronal activity in the present study is consistent to the NAc neuronal activity in response to rewarding stimuli. Previous research showed that NAc neurons were primarily inhibited by rewarding stimuli and excited by aversive stimuli (Carelli & West, 2014; Roitman, Wheeler, & Carelli, 2005; Wheeler et al., 2008). Their findings indicated that most NAc neurons showed inhibited firing rates in response to palatable (sweet solution) stimuli, but they showed an excited response after aversive stimuli (quinine). Moreover, the degree of inhibition lessened after the devaluation of the same natural reward stimuli by cocaine, the drug of abuse (Carelli & West, 2014; Wheeler et al., 2008). The results of these studies implied that NAc neurons innately encode the rewarding value and that the degree of inhibition may be correlated to its rewarding value. The present study may provide evidence the potential involvement of the endocannabinoid system on encoding rewarding value by altering NAc neuronal signal.

Moreover, inhibited NAc neuronal signaling was also found during intracranial

self-stimulation (ICSS) (Cheer, Heien, Garris, Carelli, & Wightman, 2005). In response to stimulation of medial forebrain bundle (MFB), NAc neurons showed inhibited response accompanying with dopamine release evoked by the stimulation. Even though the neuronal changes were tightly time-locked to dopamine release, NAc responses showed only subtle modulated by dopamine release but directly changed by GABA release (Cheer, Heien, Garris, Carelli, & Wightman, 2005). This study implies that dopamine in NAc may be involved in the modulation in NAc neuronal activity, but GABA release predominantly modulates it. Thus, it can be postulated that inhibited response found in this experiment may be direct effect of prefrontal input (glutamate) or local microcircuit (GABA) rather than input from VTA dopamine.

Regarding LFPs, the results showed that the power of theta rhythm was augmented after acute treatment of CB receptor activation, and this effect diminished after chronic cannabinoid treatment. Interestingly, the theta rhythm of the ventral striatum responded to the reward process, especially the learning place-reward relationship (van der Meer & Redish, 2011). Moreover, the theta rhythm of the NAc core showed a tight association with the theta rhythm of the hippocampus formation, which encodes and retrieves memory. Anatomical evidence of hippocampal projections to the NAc core also supported the functional connection. Based on these results, increased theta rhythm after acute CP55,940 may indicate an active learning process that associates place and

51

reward (cannabinoid effect) information. Moreover, diminished theta rhythm after seven days of injection may implicate a different learning phase, in which presumably a hippocampal-dependent encoding process might be less involved. Disruption of the prefrontal theta rhythm was also implicated in increased positive symptoms in schizophrenia under cannabinoid exposure (Morrison et al., 2011). This EEG study showed that oral THC altered the prefrontal theta rhythm, however, did not decrease power, but the disruption of coherence (correlation of a specific band between the electrodes) that can be an indicator of positive symptoms. Another study showed that the LFP of hippocampus and prefrontal cortex under cannabis exposure showed reduction in theta rhythm in rats (Kucewicz, Tricklebank, Bogacz, & Jones, 2011); the decreased hippocampal theta rhythm was connected to impaired working memory. Even though the power of the theta band increased in the NAc core, instead of decreasing as it did in other studies of the hippocampus and prefrontal cortex and the hypothesis, the changes in theta band could be implicated in altered neuronal functions derived from cannabinoid exposure.

Moreover, the present results suggest disassociation between neuronal activity and LFPs. Although CB receptor activation reduced NAc neuronal activities, it augmented the power of the theta rhythm in the same area. Moreover, with the CP55,940, the effect on neural activity lasted for one week during treatment. However, the cannabinoid-induced effect diminished in LFP theta rhythms during

the same time period. Moreover, CP55,940 inhibited locomotor activity after acute treatment and this cannabinoid induced decrease disappeared after chronic treatment. The cannabinoid-induced suppression in locomotion as well as tolerance development is consistent to previous behavioral studies (for review, Lichtman & Martin, 2005). In that theta rhythm of hippocampus encodes locomotion (Ledberg & Robbe, 2011), it is speculated that theta rhythm of NAc core is also possibly involved in locomotion. The tolerance development in both locomotion and NAc theta rhythm also could explain possibly connection between NAc LFP and behavior.

LFP, measurement of the extracellular current fields, was found more difficult to interpret than the activity of a single unit (Einevoll, Kayser, Logothetis, & Panzeri, 2013). Any transmembrane current (all ionic processes) from multiple sources and various properties of brain tissues contributes LFPs (Buzsáki, Anastassiou, & Koch, 2012). Although the summation of the synaptic transmembrane current (action potential) mainly affects LFPs, any inward current (and returning current) through ligand- or voltage-gated channels from the dendrites and axon terminals at the proximate site of the electrode also contribute to the power of LFPs (Einevoll et al., 2013).

LFP is continuous slower fluctuation in which the effects of the excitatory input (Na^+ and Ca^+) and inhibitory input (Cl^-) do not clearly show opposite effects. For

example, the neurotransmitters acting on NMDA receptor and AMPA receptor mediate the excitatory current (from extracellular into intracellular), creating both an inward current and an outward (passive) current to back up the ion pool leading in the opposite direction of the extracellular field. However, the inward current of ligand on GABA receptors can create the same effect of a passive current of excitatory neurotransmitters (Einevoll et al., 2013). Moreover, the different currents from different origins can cause the same effects on LFP power. Even more difficult to understand is that the geometric factor (the distance between the electrode and the origin of current change) changes the magnitude of LFP power in an inversely proportional way. Thus, LFP is more difficult to interpret than neuronal activity because of this complexity and ambiguity.

In conclusion, this dissertation study characterizes the activity of NAc core neurons in response to cannabinoid exposures. The present study provides evidence that NAc neurons primarily exhibited inhibited response under cannabinoid exposures with increased theta rhythm. Even though the response of single unit activity remained inhibited after repeated exposure, local field potentials changed under chronic exposure, suggesting that a separate mechanism was involved.

Experiment 2: Dopamine dynamics of NAc core in response to acute and chronic exposure to CP55,940

A phasic dopamine release, induced by burst firing of VTA dopaminergic neurons, mediates drug-related rewarding effects. Although acute cannabinoid administration increases spontaneous phasic DA transients in a dose-dependent manner (Cheer, et al., 2004), the previous results showed that VTA neurons could show enhanced firing rates after repeated cannabinoid treatment as being the same as the initial one (Cheer, et al., 2000; Wu & French, 2000). Therefore, it was expected that the electrically evoked DA release would increase in response to acute cannabinoid exposure and that this increase would remain unchanged after chronic exposure. However, the NAc dopamine signal in response to chronic cannabinoid exposure has not yet been elucidated. Moreover, cannabinoid effects on dopamine reuptake have brought mixed results; for example, cannabionoids inhibited dopamine reuptake in some studies (Pandolfo et al., 2011; Price et al., 2007) but failed to alter dopamine reuptake level (Cheer et al., 2004; Köfalvi et al., 2005).

Fast scan cyclic voltammetry (FSCV) technique enabled to measure evoked dopamine signals with sub-second temporal resolution. Moreover, FSCV measuring electrically stimulated dopamine overflow in anesthetized rats has been used extensively to characterized dopamine uptake and release (Addy,

Daberkow, Ford, Garris, & Wightman, 2010) because kinetic analysis on release and reuptake cannot be performed without electrically stimulated dopamine. Using FSCV to monitor evoked dopamine overflow, dopamine dynamics in the NAc core - dopamine release as well as reuptake- were investigated to determine the effect of cannabinoid exposure.

Experimental Procedures

1.Subjects

Male Sprague–Dawley rats (n= 10) weighing 250-350 g (Harlan, Indianapolis, IN, USA) were housed individually and allowed at least a week of habituation before surgery. Rat housing maintained a 12:12 h light–dark cycle (lights were turned on at 9:00 am) with water and food *ad libitum*. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Indiana University in Bloomington, IN, USA.

2. Drug Administration

The dose of 0.2mg/kg CP55,940 was selected since it induced rewarding effect measured by conditioned place preference (Braida et al., 2001a). 0.2 mg/kg of CP55,940 (Tocris, Bristol, UK) or the vehicle (10% ethanol, 10% cremophor, and 80% saline) were used for intraperitoneal [i.p.] injection.

3. Surgery

To prepare carbon fiber electrodes, a carbon fiber (7 μm in diameter, T650, Cytec, Woodland Park, NJ) was aspirated to a glass capillary (1.0 mm diameter, A-M Systems, Inc., Carlsborg, WA, USA) and pulled using a vertical micropipette puller (Narishigee, Tokyo, Japan). Under a dissecting microscope, a scalpel was used to trim the carbon fiber to a length of 120 to 150 μM from the seal of a glass and carbon fiber. The electrodes with a low background noise (the ratio of noise over the background current less than 0.001) and good shape in oscilloscope were selected and cycled with 60Hz for at least 10 minutes. A reference electrode, AgCl, was prepared from pure silver wire (0.016 inch in diameter, Scientific Instrument Services, Inc., NJ).

For the in vivo voltammetry recording, surgery was performed on the first day of injection (acute injection group) or the seventh day of injection (chronic injection group). The acute group (n=4) received a single injection of vehicle followed by an injection of CP55,940; the chronic group(n=6) received one daily injection for seven consecutive days.

On the day of surgery, rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Once the animal was mounted in the sterotaxic frame, an

incision from the anterior to the posterior of the skull was made, and the skin and fascia was removed to allow for a clear visual of the anatomical structures bregma and lambda. One hole was drilled over NAc core (+1.3 anterior and +/-1.3 lateral), and the underlying dura mater was carefully removed. Another hole was drilled over the medial forebrain bundle (MFB;-4.0 anterior and +/-1.4 lateral) on the ipsilateral hemisphere of the working electrode placed. A hole for the reference electrode was made in the contralateral hemisphere of the brain.

4. Data Acquisition

Tar Heel CV software (ESA Biosciences, Inc., Chelmsford, MA) was used to generate a triangular waveform, which ramp between -0.4V to +1.3V at a rate of 300V/S, was applied every 10ms. A house built potentiostat that controls signal transduction to a carbon fiber working electrode passes a voltage ramp to a carbon fiber electrode and measure the current at a respect to the Ag/AgCl reference electrode. The current change at the electrode compared to the reference electrode was plotted against applied potential (-0.4 to +1.3V). Dopamine is oxidized (lost two H^+ ions) approximately at +0.6V to ortho-quinone and it reduced back to dopamine approximately at -0.2V. The current change at the dopamine oxidation peak (~+0.6V) was represented to dopamine signal.

For the data acquisition, the reference Ag/AgCl electrode was placed at the hole touching to the surface of brain tissue. Then the working electrode was lowered into the NAc core; first, the electrode was lowered into 1 mm above the NAc core

and the electrode was cycled at 10Hz for 5 minutes and then targeted to the NAc core (-6.5~7.5mm below the brain surface). Finally, the biphasic-stimulating electrode (Plastic One, Roanoke, VA, 1 mm apart) was lowered to the top of the MFB (-6.5 to ~7.5 below dura). Electrical stimulation was applied (24 pulses, 60Hz, 300 µA) while stimulating electrode was optimally placed along the dorsal-ventral axis. The MFB was located at 1-1.5 mm below the point at which showed a weak whisker and/or foot twitch. Once stimulation parameters were optimized, the biphasic (24 pulses, 60Hz, 300 µA) stimulation was applied every 2 min.

For the acute group, five consecutive trials were averaged to represent the evoked dopamine concentration in the baseline state (pre-treatment). After that, vehicle was injected another five consecutive trials were averaged to represent the evoke dopamine concentration in response to acute vehicle injection. The same group of animals of the vehicle injection received 0.2 mg/kg of CP55,940. Five consecutive trials were averaged every 10 min and the highest averaged current was selected to represent the evoke dopamine concentration after the CP55,940 injection.

For chronic vehicle or CP55,940 group, same as the acute group, five consecutive trials were averaged to represent the evoked dopamine concentration in the baseline state (pre-treatment). After that, animals, who received six days of vehicle (chronic vehicle group) or CP55,940 injections

59

(chronic CP55,940 group), received the seventh injection of vehicle or CP55,940, respectively. Five consecutive trials was averaged every 10 min and the highest averaged current was selected to represent the evoke dopamine concentration after the CP55,940 injection.

5. Post Calibration of the electrode

The current amplitude *in vivo* is considered to be proportional to the concentration of dopamine. The evoked dopamine concentration was calculated with either direct post-calibration or post-calibration curve. For the post-calibration, immediately after the data acquisition, the electrode was carefully removed from the rat brain and was placed in the Tris buffer (12 mM tris–HCl, 140 mM NaCl, 3.25 mM KCl, 1.2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2 mM $NaSO_4$, pH = 7.4) for post-calibration. In a flow cell system, Tris buffer was constantly streamed and the switch for the 1 μM dopamine was manually on/off. The voltammetric signal of the pulse of 1 μM dopamine was obtained (Fig 24). The current (represented in color) was plotted against the applied voltage (-0.4 to 1.3V) and dopamine was detected at its oxidation peak (~+0.6V, green color) during the electrode was exposed to dopamine solution. The voltammogram (Fig 24, right panel) showed that the chemical signature of dopamine which oxidized at ~+0.6V and reduced at ~-0.2V. Further, the signal was transited to the concentration using the current/concentration ratio to calculate the evoked dopamine concentration on the electrode. The post-calibration curve was also

used to convert dopamine current to concentration. For the post-calibration curve, the background known dopamine concentration were plotted and the estimated linear response was calculated.
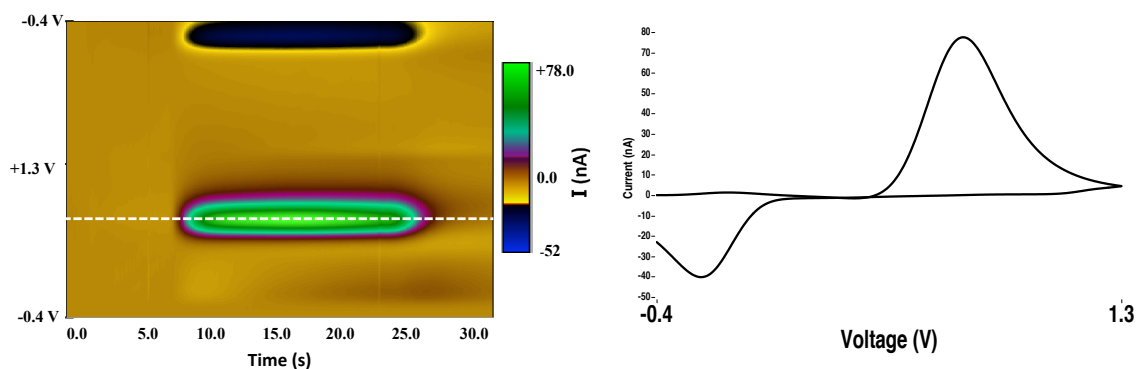
Figure 24. An example of color plot (left) and cyclic voltammogram (right) of dopamine solution in the flow cell system. In the color plot, x-axis represents time (s), the y-axis represents applied potential to the electrode (V) and current was shown in color (nA, scale on right). The electrode was exposed for ~ 10 s and dopamine was detected at its peak of oxidation (~ +0.6 V, dashed white line). Cyclic voltammogram showed the chemical signature of dopamine that is oxidized at ~+0.6V and reduced at ~-0.2V.

6. Data Analysis

- Dopamine concentration

The percentage change compared to the baseline (% of baseline) were calculated in response to the injection of CP55,940 or vehicle. An independent samples t-test was performed to compare the evoked dopamine concentration (% of baseline) between vehicle and CP55,940 treatment. One-way ANOVA with Bonferroni's multiple comparisons was performed to compare the baseline

evoked dopamine concentration of the three conditions; acute group (no treatment), chronic vehicle, and chronic CP55,940.

-Mathematical modeling

Dopamine release and reuptake were estimated from Michaelis-Menten kinetics and exponential fit modeling developed by Demon Voltammetry analysis software (Wake Forest University, Winston-Salem, NC). Briefly, the Michaelis-Menten equation for dopamine uptake can be written by

d[DA]/dt=[DA]p-Vmax/(Km/[DA]+1)

[DA] is the extracellular concentration of dopamine, [DA]p is the change of dopamine release per stimulus, Vmax and Km are Michaelis-Menten constant on dopamine reuptake by dopamine transporter. Vmax, which is directly propotional to DAT concentration, stayed constant throughout the recording session. The Km value, which shows the affinity of DAT at half value of Vmax, was set as 200nM in the baseline. Demon Voltammetry software was used to fit the data into the Michaelis-Menten equation. The Michaelis-Menten kinetics assume that (1) each stimulus pulses release a fixed amount of dopamine from the presynaptic terminals; (2) clearing dopamine mechanism is mainly dependent on uptake via the DAT; (3) uptake is a saturable process with limited affinity/concentration of DAT (Wightman et al., 1988; Yorgason, España, & Jones, 2011). The percentage

changes compared to the baseline (% of baseline) were calculated. An independent samples t-test was performed to compare the calculated Km and [DA]p (% of baseline) of each condition; vehicle vs. CP55,940 in the acute and chronic condition.

Exponential fit modeling provided by Demon Voltammetry software was also used for further mathematical modeling for the dopamine release and uptake. In this analysis, baseline (pre-stim cursor), peak (peak cursor) and return to baseline (post-stim cursor) were defined (Fig 25 (a)). Briefly, Tau and half-life were calculated from exponential fit curve determined by peak cursor and post-stim cursor position using a least square controlled exponential fit algorithm (Fig 25 (b)). Area Under Curve (AUC) was calculated as the numeric integration of the area between the pre-stim and post-stim cursors. Full width at half height (FWHH) was obtained by measuring the time between the rise and decay portions at the half-maximal amplitude based on pre-stim cursor and post-stim cursor. Tau, FWHH, half-life showed strong correlation which changes in Km and reliable measures for detecting changes in DA uptake (Yorgason et al., 2011).
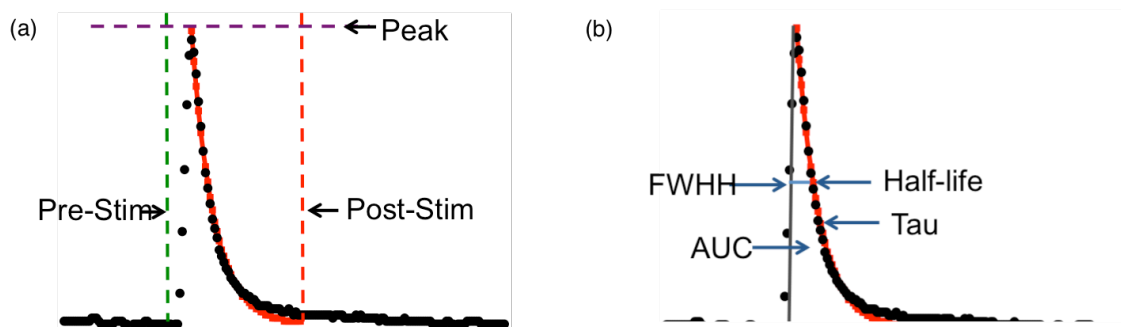
Figure 25. Measures of release and reuptake. (a) Positions of Peak (Purple dashed line), Pre-stim (Green dashed line), and Post-Stim (Red dashed line)

(b)Half-life, Area under Curve, Tau and FWHH(Full width at half height) base on the location of Pre-stim, Post-stime and Peak

Results

1. **Evoked dopamine signal**

To examine the effect of CP55,940 on the dopamine overflow, evoked dopamine signal was obtained using FSCV in anesthetized animals. Figure 24 shows examples of color plots and concentration-time plots of baseline and acute CP55,940 injection; the concentration-time plots (Fig 26 (a)) showed the current changes at ~+0.6V (white dashed line in Fig 26 (b)) in response to the applied electrical stimulation (red bar below the concentration-time plot). The current (represented in color) was plotted against the applied voltage (-0.4 to 1.3V) and dopamine was detected at its oxidation peak (~+0.6V, green color) during the electrical stimulation. Cyclic voltammogram (inset of the concentration-time plot) showed the oxidation peak at ~ +0.6 V and reduction peak at ~ -0.2 V that is identical to the chemical signature of dopamine.

(a) Concentration versus time with voltammogram

Baseline

Acute CP55,940

(b) Color plot

Baseline
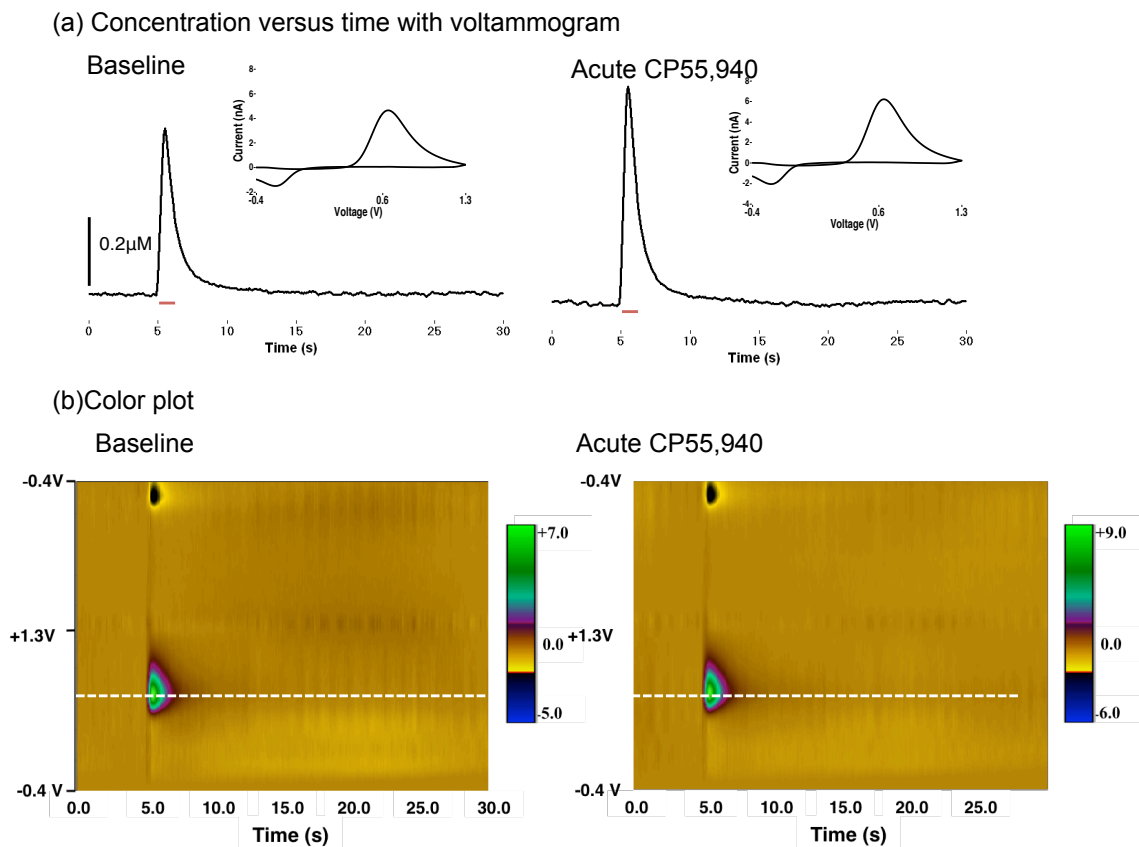
Acute CP55,940

Figure 26. Concentration versus time plot (upper panel) with voltammogram (inset) and representative color plots (lower panel) before the treatment (a) after acute injection of CP55,940 (b). (a) Concentration-time plots represent the concentration of dopamine over time. Red bar below the plot indicate the duration of electrical stimulation. Acute injection of CP55,940 increased dopamine concentration. Cyclic voltammogram (inset) showed the chemical signature of dopamine that is oxidized at $\sim+0.6V$ and reduced at $\sim-0.2V$. (b) In the color plot, x-axis represents time (s), the y-axis represents applied potential to the electrode (V) and current was shown in color (nA, scale on right). Dopamine was detected at its peak of oxidation ($\sim +0.6$ V, dashed white line, green color).

The acute CP55,940 treatment (0.2mg/kg, i.p.injection) significantly increased the evoked dopamine concentration (% of baseline) compared to the acute vehicle treatment (independent t-test, $p<.05$, Fig 27, Table 3). The acute CP55,940 treatment increased the dopamine concentration about 20% compared to the baseline (pre-treatment). The chronic CP55,940 treatment did not show a significant difference in evoked dopamine concentration (% of baseline) compared to the chronic vehicle treatment. However, the concentration changes in the chronic CP55,940 treatment was significantly lower than that of the acute CP55,940 treatment (independent t-test, $p<.05$).

Table 3. Percentage changes from baseline in evoked dopamine signal following acute or chronic treatment with vehicle or CP55,940

Group	% of baseline (±SEM)
Acute vehicle	93.87 % (± 6.851)
Acute CP55,940	119.7 % (± 6.647)
Chronic vehicle	106.3 % (± 15.28)
Chronic CP55,940	92.77 % (± 4.721).
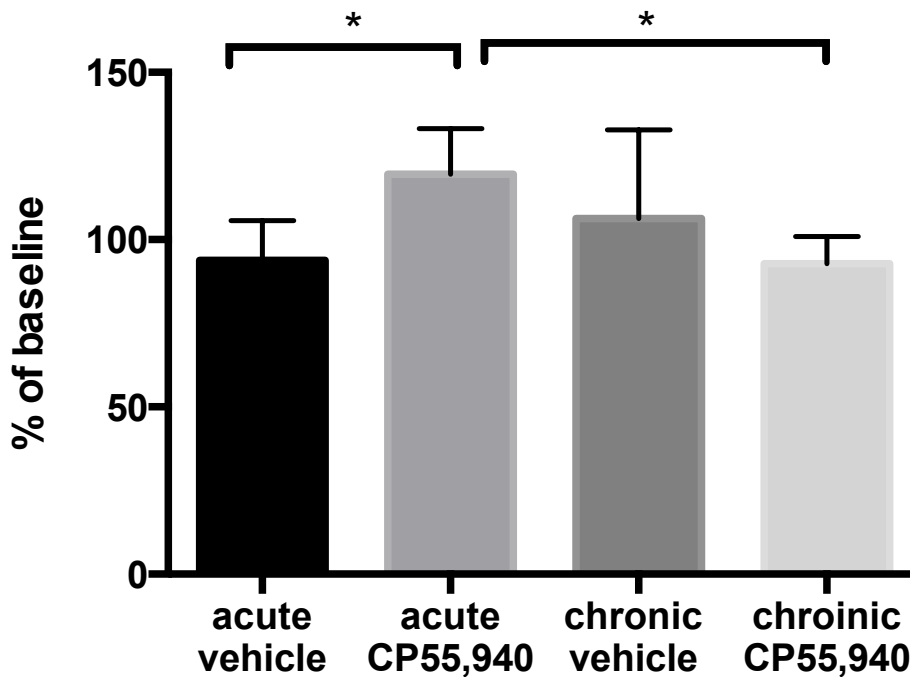
Figure 27. Percentage changes from baseline in the evoked dopamine signal following acute or chronic exposure of vehicle or CP55,940 Acute CP55,940 (0.2mg/kg, i.p.) significantly increased evoked dopamine concentration compared to acute vehicle injection. Chronic CP55,940 did not show a significant change in evoked dopamine concentration compared to both vehicle groups, but Chronic CP55,940 was significantly lower than acute CP55,940. Error bar represents SEM, independent sample t-test, *p<.05

2. Pre-injection evoked dopamine response

Averaged evoked dopamine concentration before either vehicle or CP55,940 treatment were compared by one way ANOVA with Bonferroni's multiple comparisons. There were no significant differences across the groups. The mean evoked dopamine concentration was 0.3559 µM (± 0.1082), 0.4742 µM (±

0.1801), and 0.1013 µM (±0.0337) for the acute group, chronic vehicle group, and chronic CP55,940 group, respectively.

3. Michaelis-Menten modeling

1)[DA]p – dopamine release per stimulus

The modeled [DAp], the amount of the dopamine concentration by each pulse, showed a significant increase after acute CP55,940 injection compared to vehicle injection and chronic CP55,940 (Fig 28, Table 4). An independent t-test confirmed the significant difference between the acute CP55,940 vs. the acute vehicle as well as the acute CP55,940 vs. the chronic CP55,940 treatment ($p<.05$).
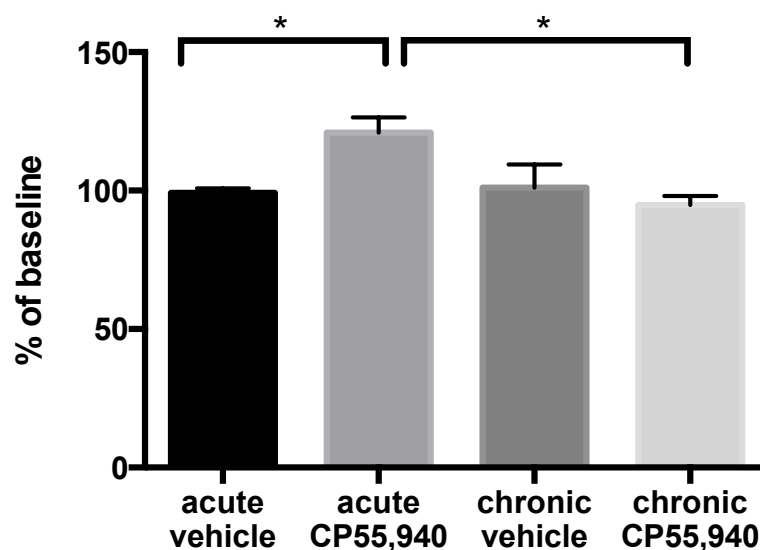
Figure 28. [DA]p of Michalis Menten modleing. A significant increase in [DA]p, dopamine release per stimulus pulsewas found in acute CP55,940 treatment

when compared to acute vehicle and chronic CP55,940. Error bar represents SEM, independent sample t-test, *p<.05

Table 4. The percentage changes (% of baseline) in the [DA]p following acute or chronic exposure of vehicle or CP55,940

Group	% of baseline (±SEM)
Acute vehicle	99.17 % (± 1.688)
Acute CP55,940	121.0 % (± 5.477)
Chronic vehicle	101.1 % (± 8.284)
Chronic CP55,940	94.84 % (± 3.249)

2) Km – dopamine reuptkae

Along with the dopamine release ([DAp]), the amount of dopamine reuptake by dopamine transporter was modelled. The Km value in Michaelis Menton kinetic represents dopamine transfer function at ½ Vmax, serving as a measure of dopamine transporter efficiency (Johnson, Rajan, Miller, & Wightman, 2006). The modelled Km value showed that the CP55,940 treatments, both acute and chronic treatment, were not significantly different from the vehicle treatments. The Km values after the treatments did not significantly differ from the Km value in the baseline.

4. Exponential Fit Modeling

1) Area Under Curve (AUC)

Acute CP55,940 treatment significantly increased the AUC compared to the acute vehicle injection (independent t-test, $p<.01$) (Fig 29, Table 5). Compared to the baseline, acute CP55,940 treatment increased the AUC by about 20% that was consistent to the changes in dopamine concentration. The chronic CP55,940 treatment did not show a significant difference in the AUC (% of baseline) compared to the chronic vehicle treatment. However, the AUC of the chronic CP55,940 treatment was significantly lower than that of the acute CP55,940 treatment (independent t-test, $p<.01$, Fig 29, Table 5).
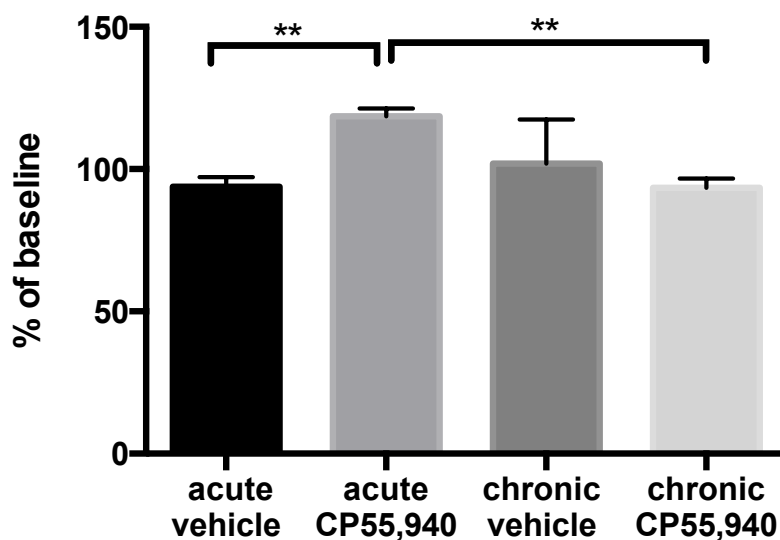
Figure 29. Changes in AUC (% of baseline) after acute and chronic treatment. A significant increase in AUC (Area Under Curve) was found in acute CP55,940 treatment when compared to acute vehicle and chronic CP55,940 Error bar represents SEM, independent sample t-test, **$p < .01$

Table 5. The percentage changes (% of baseline) in the AUC following acute or chronic exposure of vehicle or CP55,940

Group	% of baseline (±SEM)
Acute vehicle	93.81 % (± 3.379)
Acute CP55,940	118.5 % (± 2.757)
Chronic vehicle	102.0 % (± 15.43)
Chronic CP55,940	93.43 % ± (3.302)

2) Tau

Tau, decay time constant, slightly increased after acute CP55,940 treatment compared to the acute vehicle injection, however, the difference between two groups was not significant; the change in Tau values relative to the baseline were 104.9 % (±12.22) for acute vehicle and 111.4% (±11.42) for acute CP55,940 treatment. Also, there was no significant difference between the chronic vehicle versus the chronic CP55,940 groups; 98.78 (±5.454) for the chronic vehicle treatment; 97.37% (±4.233) for the chronic CP55,940 treatment.

3) Full width at half height (FWHH)

FWHH, a time between rise and decay portion of the trace at half value of the peak, was obtained and compared across the groups in acute and chronic treatment. Acute CP55,940 treatment slightly increased the value of FWHH slightly, but it was not statistically significant (independent sample *t* test); 104.1% (± 14.01) for acute vehicle treatment and 122.7% (± 6.316) for acute CP55,940 treatmen. Also, there was no significant difference between the chronic vehicle versus the CP55,940 groups; 103.6% (± 2.487) for the chronic vehicle treatment; and 96.15% (± 2.071) for the chronic CP55,940 treatment.

4) Half-life

Like a Tau, the half-life (= the time when the peak decay by one half) slightly increased after acute CP55,940 treatment compared to vehicle treatment but it was not significant. The change of half-life value relative to the baseline was 103.1% (± 13.85) for the acute vehicle treatment and 111.4% (± 11.42) for the acute CP55,940 treatment Also, there was no significant difference between the chronic vehicle versus the CP55,940 groups; 98.78% (± 5.454) for the chronic vehicle treatment; and 97.37% (± 4.233) for the chronic treatment.

Discussion

To the best of my knowledge, this voltammetry study offers the first *in vivo* characterization of dopamine dynamics on the NAc core under acute as well as chronic cannabinoid treatment. Using FSCV and kinetic analysis, this study measured both release and reuptake elements in the dopamine signal in the NAc core, where dopamine transporters are more abundantly expressed than shell (Nirenberg et al., 1997). Acute treatment of CP55,940 increased the evoked dopamine concentration by 20% relative to baseline, and this effect disappeared after the seventh day of injection.

This result suggests that acute cannabinoid exposure increases dopamine overflow in the NAc core mainly due to an increase in evoked dopamine release, not by inhibition in dopamine reuptake. The increase in evoked dopamine release did not last after one week of daily cannabinoid treatment, implying that there are mechanisms for developing tolerance. The kinetic analysis of dopamine overflow suggests that [DA]p, dopamine release per stimulus, and AUC (area under curve), were significantly increased in response to acute CP55,940 treatment. The degree of increase in AUC corresponds to that of the evoked dopamine concentration (both 20%) and those increase rates are supported by the 20% increase in [DA]p level.

Moreover, neither acute nor chronic treatment of CP55,940 (0.2mg/kg, i.p.) altered the measurement of dopamine reuptake; Km, Tau, Full width at half height (FWHH), and half-life. Km value in the Michaelis-Menten equation and Tau, FWHH, and half-life value obtained from mathematical modeling are measurements of dopamine reuptake levels and showed strict correlations between those values (Yorgason et al., 2011). In that those values did not show significant difference compared to the baseline, we can propose that cannabinoid treatment did not alter dopamine transporter function.

The result suggests that cannabinoids increased dopamine release rather than altered dopamine transporter functions. An FSCV study showed that intravenous cannabinoid injection increased dopamine transient in NAc in freely moving rats (Cheer et al., 2004). Also, this study suggested that cannabinoids may not be involved in the reuptake because clearance rates remained unchanged after cannabinoid treatment. Moreover, an *in vitro* study also supported that cannabinoids did not change the reuptake level of dopamine in striatal slices (Köfalvi et al., 2005). However, other studies suggested the opposite; cannabinoids can alter the reuptake rate by inhibiting dopamine transporter function. Price and his colleagues examined dopamine clearance under cannabinoids using *in vivo* and *in vitro* assay and concluded that cannabinoids could inhibit dopamine reuptake (Price et al., 2007). A similar study also suggested that that both endogenous and exogenous cannabinoid receptor

agonists interacted with the dopamine transporter and inhibited the reuptake process (Pandolfo et al., 2011). Both studies have in common that they measured reuptake level in synaptosomes in rodent striatum as well as the inhibition of dopamine transporter being independent upon cannabinoid receptor function.

The discrepancy in the cannabinoid induced effect on dopamine transporter function may come from 1) different brain regions: NAc (this dissertation work and Cheer's work vs. entire (Kofalvi's work) or dorsal striatum (Pandolfi's work); 2) different techniques for measuring reuptake: *in vivo* (the dissertation work and Cheer's work) and *in vitro* for others. Even though Price and his colleagues provide *in vivo* technique (high-speed chronoamperometry) in anesthetized animals, the differences between the FSCV and high-speed chronoamperometry may create the inconsistent results in terms of time scale of measurement (subsecond vs. minute).

One of the potential mechanisms underlying the observed increase in rapid dopamine release after acute cannabinoid treatment is increased burst firing rates of VTA dopaminergic neurons (Cheer, Marsden, Kendall, & Mason, 2000b; Wu & French, 2000). This increased VTA dopaminergic neuronal firing is further supported by inhibition of GABA release to VTA neurons under cannabinoid exposure (Lecca, Melis, Luchicchi, Ennas, Castelli, Muntoni, et al., 2011a). A

77

previous FSCV study in freely moving animals also reported an increased level of dopamine transient which was blocked by CB receptor antagonist (Cheer, Marsden, Kendall, & Mason, 2000a). Furthermore, the possible role of cannabinoid on facilitating dopamine release in the dopaminergic terminals will be another potential underlying mechanism increasing dopamine signals. Recent finding suggests that cannabinoid modulates dopamine release by interacting with adenosine receptor (Ferré et al., 2010). Moreover, another finding indicates that cannabinoids increase dopamine signal by inhibiting dopamine monoamine oxidase (Fišar, 2012). Thus, in addition to increasing dopaminergic neuronal firing, emerging evidence also shed light on a possible role of cannabinoid in the dopaminergic terminals as well.

Moreover, the present results indicate that the cannabinoid-induced changes in dopamine overflow develop tolerance after repeated treatments. A recent finding using FSCV also suggests that repeated cannabinoid exposure reduced dopamine releasing effects (Oleson, Ranganath, Karamsetty, & Cheer, 2014); repeated WIN treatment caused tolerance to cannabinoid-induced behavior effect well as dopamine releasing effect. In that down-regulation of CB1 receptor is thought to be involved in the development of cannabinoid tolerance (González, Cebeira, & Fernández-Ruiz, 2005; Martin et al., 2004), repeated treatments in the present study may return CB1 receptor signaling back to the baseline, presumably by reducing CB1 receptor function.

Overall, by using FSCV and kinetic analysis to separate release and uptake components of the dopamine overflow, this dissertation work provides characterization of *in vivo* dopamine dynamic under cannabinoid exposure. Our results show that although CB1 receptor activation increases NAc dopamine concentration and tolerance develops to this effect with chronic treatment. Thus, altered dopamine signaling in the reward pathway by acute and chronic cannabinoid exposure may contribute to differential changes in motivational state

General Discussion

As a part of the mesolimbic pathway, NAc modulates emotional and motivational states. The manipulation of CB1 signaling within the NAc has been found to cause robust changes in emotional and motivational states related to drug addiction and other psychopathological conditions (Winters et al., 2012). The research conducted for this dissertation examined the neuronal adaptation in the NAc core under cannabinoid exposure. The study examined two main changes in the NAc core: neuronal activity and dopamine dynamics under the acute and repeated activation of CB receptors. The main finding suggested that cannabinoids reduced neuronal signaling and disrupted functional communication, which lasted after repeated treatment. Moreover, the cannabinoids increased the evoked dopamine release without altering the dopamine reuptake, but this effect disappeared after repeated treatment. Because CB receptor activation attenuates presynaptic excitatory and inhibitory input, the examination of all components of the CB receptor could help in understanding the functional output of CB receptor activation.

Based on the presented results, neuroadaptation of NAc neuronal signaling and dopamine dynamics can be potentially explained by two separate adaptive mechanisms; glutamate projection to the NAc core vs. dopaminergic projection to the NAc core. Consistent with the previous studies, the present study confirms

cannabinoid reduced NAc neuronal activity presumably by inhibiting presynaptic glutamate release (Mato et al., 2005; Robbe et al., 2001). The inhibited glutamate release is possibly involved in the reduced neuronal activity found in the present study. Moreover, increased evoked dopamine release in this present study also can be explained by CB receptor induced disinhibition of VTA dopaminergic neurons (Oleson et al., 2012). Moreover, increased excitability and burst firing of dopaminergic neurons promote the endocannabionid release which further facilitate CB receptor mediated disinhibition of VTA (Fitzgerald, Shobin, & Pickel, 2012). Excitatory glutamatergic input from the prefrontal cortex and dopaminergic input from VTA projects are assumed the underlying mechanisms of the altered neuronal signal and dopamine dynamic in the present study. In fact, the interaction of these two projections in the NAc is required for behavioral adaptation in goal directed behavior (Pennartz et al., 1994; Wang et al., 2012).

The discrepancy in cannabinoid induced tolerance between neuronal signal and dopamine dynamic in NAc core implies a regional difference in the development of tolerance. Chronic exposure of cannabinoid reduced CB receptor signaling, and these changes were closely associated with cannabinoid induced behavioral effects (González et al., 2005; Martin et al., 2004). The CB1 down-regulation is larger and takes place more rapidly in the cortical area than in the subcortical area including basal ganglia and midbrain (Martin et al., 2004)). In fact, a recent human study clearly revealed that chronic marijuana smoking showed

significantly lowered CB1 receptor density in the midbrain but not in striatum (Hirvonen et al., 2012). This regional difference may provide evidence as to why NAc dopamine signaling (Midbrain CB receptor involved) develops tolerance whereas NAc neuronal signaling (NAc CB receptor involved) does not.

In addition to the regional difference, various cannabinoid effects show different behavioral aspect of tolerance. For example, tolerance for memory impairment develops rapidly, but not for feelings of euphoria in humans (D'Souza et al., 2008). In animal studies, repeated administration of cannabinoids diminish cannabinoid effects on responses in locomotion, antinociception, and hypothermia (Maldonado, 2002). It is not fully understood how the changes in rewarding effects of cannabinoids function. The lower tolerance for feeling high might be supported by the present finding of persistently decreased NAc neuronal signaling; persistent reduction in NAc core signaling may reduce inhibitory NAc input to the VTA, which might play a role in maintaining increased VTA neuronal excitability after repeated cannabinoid treatment. Consistently enhanced VTA neuronal firing after repeated cannabinoid administrations in previous studies (Cheer, Marsden, Kendall, & Mason, 2000a; Wu & French, 2000) are therefore supported by the present finding showing steady NAc firing inhibition under chronic cannabinoid treatment.

Consistently enhanced VTA neuronal firing in previous studies may contradict to

the present voltammetry results. If the VTA dopaminergic activity is sustained consistently, it can be expected that evoked dopamine release also remains unaltered after repeated cannabinoid treatment. However, it is also possible that evoked dopamine release can be regulated primarily in the dopaminergic terminal rather in the cell body. Applied frequency of stimulation (60Hz) is higher than burst rate of dopaminergic neuron (~20Hz) and could dominate the cannabinoid-induced effect on increasing dopaminergic neuronal activity.

This dissertation study chose non-contingent drug exposure (i.p. injection) that potentially recruits different neuronal mechanisms than those involved in volunteer drug taking. Moreover, neuronal activities measured in freely moving animals examined the spontaneous neuronal discharge under cannabinoid exposure. However, the dopamine dynamic was measured in anesthetized animal for the precise kinetic modeling process, which separates dopamine release and reuptake portions. This study proposed to examine dopamine and glutamate interplay in the NAc core, but further consideration is required to interpret because neuronal activity and dopamine dynamics measured in different waking states (freely moving condition vs. anesthetized condition). Moreover, even though the dose used in this study is assumed to intrigue rewarding effects based on previous studies, lacks behavioral measurements that examine actual rewarding properties of cannabinoid was one of the limitations of this study. Behavioral measurement that concerns the development of cannabinoid induced behavioral tolerance potentially support NAc signal change found in this study.

Cannabinoids emerge as a therapeutic target for treatment of depression (Segev, Rubin, Abush, Richter-Levin, & Akirav, 2014), drug addiction (López Moreno, González Cuevas, Moreno, & Navarro, 2008) , and movement disorder (van der Stelt & Di Marzo, 2003). Veiled side effects or tolerance development with repeated use limits the cannabinoid for therapeutic use. This dissertation work provides particularly the potential effects of cannabinoids on NAc core, a key component of the rewarding pathway. As our results indicate, cannabinoids inhibited NAc core neuronal activity during initial and prolonged treatment, which could provide evidence of changes in motivational states that, in turn, could play a role in changing reward-related behaviors. Cannabinoid induced alterations presented in this study can further explain the underlying mechanism of NAc mediated behavioral adaptation in the decision making process.

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EDUCATION

Indiana University Bloomington, IN, USA
- Ph.D., Neuroscience, February 2015
- Ph.D. Dissertation: Neuronal Adaptations in the Reward Pathway under Acute and Chronic Exposure to Cannabinoids (advisor: Dr. George V. Rebec)

Seoul National University, Seoul, South Korea
- M.S., Neuroscience, 2005
- Master's Thesis: Stress and Cognitive Aging (advisor: Dr. Jeanyung Chey**)**

Seoul National University, Seoul, South Korea
- B.S., Biology, 2003

RESEARCH EXPERIENCE & SKILLS

Indiana University Bloomington, IN, USA
Graduate Student (Ph.D.) with Dr. George V. Rebec (08/2008-02/2015)
Examined the neuronal/behavioral changes in response to cannabinoids exposure in adolescent and adult rats
Skills: in vivo electrophysiology in freely moving rats (aseptic surgery to implant electrodes and analyze neuronal signals), fast-scan cyclic voltammetry (surgery and detecting dopamine signals), rodent brain dissection and western blot assay, rat behavioral assessment including Go/No-Go task, nose-poke impulsivity test, novelty task, open field locomotion

Indiana University Bloomington, IN, USA
Research Assistant (01/2007-07/2007),
Graduate (Ph.D.) Student with Dr. J. Michael Walker (08/2007-05/2008)
Developed a HPLC/MS/MS (liquid chromatography-tandem mass spectrometry) method for detecting farnesyl pyrophosphate (FPP) and examined its distribution in rat brains
Skills: Extracted and analyzed the level of FPP using HPLC/MS/MS, Brain dissection

Indiana University-Purdue University Indianapolis, IN, USA
Research Assistant (08/2006-12/2006) with Dr. Younglim Lee
Assisted the project titled, "Development of Stress Resilience: Can CRF R2 Activation Counteract CRF R1-induced Stress Vulnerability"
Skills: Cannula implantation surgery/cannula injection/behavioral test (social interaction test)

Seoul National University, Seoul, South Korea
Research Assistant (03/2005-03/2006) with Dr. Jeanyung Chey
Designed and conducted a longitudinal study titled, "Study on the mechanism of memory disorder/prefrontal dysfunction and development of cognitive improvement technology."

<u>Skills</u>: Assessment of the neuropsychological test of K-DRS (Korean version of Dementia Rating Scale) and EMS (Elderly Memory Scale), neuroimaging data analysis using SPM (Statistical Parametric Mapping) and Matlab

OTHER EDUCATION

The Summer Institute of the International Neuropsychological Society "The Human Frontal Lobe" Xylocastro, Greece (06/2004-07/2004)

PUBLICATIONS

1. <u>Lee, S</u>. and Rebec, G. (in preparation). "Neuronal adaptations in the nucleus accumbens core under acute and chronic exposure to cannabinoids"
2. Ahn, W.-Y., Vasilev, G., <u>Lee, S.</u>, Busemeyer, J. R., Kruschke, J. K., Bechara A., & Vassileva, J. (2014)"Decision-making in stimulant and opiate addicts in protracted abstinence: evidence from computational modeling with pure users. *Frontiers in Decision Neuroscience*, 5:849
3. <u>Lee, S</u>, S. Raboune, S., Walker, J. M., & Bradshaw, H. B. (2010). "Distribution of endogenous farnesyl pyrophosphate and four species of lysophosphatidic acid in rodent brain." *International journal of molecular sciences*, *11*(10), 3965-3976.
4. Bradshaw, H. B., <u>Lee, S.</u>, & McHugh, D. (2009). "Orphan endogenous lipids and orphan GPCRs: a good match." *Prostaglandins & other lipid mediators*, *89*(3), 131-134.

POSTER PRESENTATIONS

1. <u>S. Lee</u>, K. Bunner, and G. Rebec, "Evoked dopamine dynamics in nucleus accumbens core following acute versus chronic administration of the CB1/CB2 agonist, CP55,940" *Society for Neuroscience* (2014)
2. <u>S. Lee</u> and G. Rebec, "Neuronal adaptations of the nucleus accumbens core to acute vs. chronic exposure to the cannabinoid receptor agonist, CP55,940" *Society for Neuroscience* (2012)
3. <u>S. Lee</u> and G. Rebec, "Chronic treatment with cannabinoid receptor agonist, CP 55,940, alters GLT1 expression in adolescent rats" *Society for Neuroscience* (2010)
4. <u>S. Lee</u>, B. Tan, J. M. Walker, and H.B. Bradshaw, "Farnesyl Pyrophosphate, a potential mediator of pain and inflammation, is most abundant in the brainstem, midbrain, and thalamus of the rat" *Society for Neuroscience* (2008)
5. <u>S. Lee</u>, J. Chey, B. Cho, S. Kim, Y. Kwak, "Physiological Stress Response is Associated with Increased Regional Glucose Metabolism in Anterior Cingulate Cortex and Poor Episodic Memory in Normal Elderly Adults" *International Neuropsychological Society* (2007)
6. J. Chey, <u>S. Lee</u>, Y. Kwak, H. Kim, J. Lee, M.Yang, "Stress and Cognitive Aging in Healthy Elderly Populations" *The Annual Meeting of the Korean Society for Brain and Neural Science* (2006)
7. <u>S. Lee</u>, Y. Kwak, J. Chey, "The Range of Basal Cortisol Associated with Decreased Glucose Metabolism in Middle Temporal Lobe and Lower Declarative Memory Performance" *Society for Neuroscience* (2005)

8. Y. Kwak, S. Cho, <u>S. Lee</u>, Y. Kim, W. Lee, J. Chey, S. Kim, "Relationship Between Salivary Cortisol Levels and Regional Cerebral Glucose Metabolism in Non-demented Elderly Subjects" *Society of Nuclear Medicine* (2005)

HONORS & FELLOWSHIPS

1. MetaCyte Research Fellowship by Eli Lilly Corporation (USA): <u>08/2007 – 08/2010</u>
2. Travel Award by College of Arts and Sciences Graduate Program, Indiana University (USA): <u>Fall 2008</u>
3. Honors Scholarship by Seoul National University (South Korea): <u>Fall 1999-Spring 2000, Fall 2001-Spring 2002, Spring 2004</u>
4. Summer Institute Student Scholarship by International Neuropsychological Society: <u>06/2006-07/2004</u>
5. Research Assistantship by Brain Research Center (South Korea): <u>12/2003-3/2006</u>