UNIVERSITY OF CALIFORNIA, SAN DIEGO

Inhibition of Protein Kinase C Intracerebroventricularly Attenuates Sensitization

A thesis submitted in partial satisfaction of the

requirements for the degree Master of Science

in

Biology

by

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2012

The thesis of Oliver Daniel Mrowczynski is approved, and it is acceptable

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Chair

University of California, San Diego

2012

I dedicate this thesis to Gisela, Andreas, and Brittney for their continuous love and support throughout this whole process.

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Acknowledgments

First and foremost, I would like to thank Dr. Stephan Anagnostaras for the opportunity and all the invaluable experience I have attained by working in his lab. Spending time in his lab has opened my eyes to the wonders of research and has led me on not only the MD path I had once wanted, but now on the path of dual combined MD/PhD.

I would also like to thank Kristin Howell for all of her help and training in the surgical, laboratorial, and statistical techniques used for my project. All of her time and effort spent being my mentor and guiding me through trouble shooting and experimental design was paramount to my success, and for that I am truly thankful.

Next, I would like to thank all of the members of the Anagnostaras lab, including but not limited to Kleou, Tristan, Stephanie, Brad, and Mike, for all of their support and contributions throughout.

I would like to thank Dr. Gentry Patrick and Dr. Jill Leutgeb for their effort and time being part of my esteemed Master committee.

Lastly, I would like to thank my friends and family for their continuous love, encouragement, and support during my years as a graduate student and throughout this project.

ABSTRACT OF THE THESIS

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by

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Master of Science in Biology

University of California, San Diego, 2012

Professor Stephan Anagnostaras, Chair

Drug relapse, mediated by drug-associated memories, is a major problem associated with addiction. Protein kinase C (PKC) is a family of protein kinase enzymes that has been implicated in learning and memory with regards to addiction. This study used a PKC inhibitor, chelerythrine (10nmol), to investigate the effects of blocking PKC throughout the brain on addiction related memories. Cocaine (15mg/kg) induced locomotor sensitization, used to model the transition from casual to compulsive use, and conditioned place preference, used to model drug seeking behavior, were investigated. Chelerythrine or aCSF was delivered continuously throughout the experiment through the use of an osmotic mini pump. Chelerythrine infused mice did not have any significant change of preference in comparison to the aCSF controls. Twenty-four hours after the preference test, all mice received an injection of cocaine (15mg/kg) and locomotor sensitization was analyzed. It was found that experimental mice had a significant negation of sensitization in comparison to the controls.

I.

Introduction

Drug relapse, mediated by drug-associated memories, is a major problem associated with addiction (Hunt et al., 1971). Previous studies have shown that this relapse is mediated through environmental cues that are linked to drug effects and drug use (Wilker et al 1973, O'Brien et al 1986). These environmental cues are modeled through drug addiction paradigms such as conditioned place preference and sensitization.

Conditioned place preference (CPP) is a classical conditioning procedure which contains a training phase in which one environmental context is paired with drug injections, while another context is paired with vehicle (e.g., saline) injections (Mucha et al 1982). During subsequent drug-free CPP tests, mice choose between the drug- and vehicle-paired contexts. Increased preference for the drug-paired context serves to measure the drug's rewarding effects and drug seeking behavior (Mucha et al., 1982; Stewart, 1992). This behavior was also shown in laboratory mice: cues previously associated with drug intake also provoke drug seeking after prolonged abstinence (Grimm et al., 2001; Ciccocioppo et al., 2004).

The sensitization paradigm serves as a model for the transition of casual to compulsive drug use in addicts (Shuster et al 1977). Psychostimulant use produces behavioral sensitization which is characterized by increased stereotyped behavior and locomotion (Robinson & Becker, 1986; Segal & Kuczensk, 1994;Yamanaka, Yamamoto, & Egashira, 1983). Sensitization is also accompanied by mesolimbic dopamine transmission changes mediated through changes in neuroplasticity. (Robinson & Berridge, 1993, 2000). This was shown in previous studies in which repeated administration of cocaine to mice increased their locomotion response as much as four-fold over the response to the first injection (Shuster et al 1977).

Sensitization to the behavioral effects of cocaine was more pronounced following drug administration in the presence of cues previously associated with cocaine administration than in their absence. This indicates that Pavlovian conditioning contributes to sensitization, and has implications for treatment of stimulant abuse (Hinson et al 1981).

Protein kinase C (PKC) comprises a family of kinases that regulate a variety of nervous system functions, including cellular communication, cytoskeletal reorganization, and neuronal housekeeping via phosphorylation of a myriad of substrates (Brennan et al 2009). More than 10 isoforms of PKC have been identified, and the majority of these isoforms are present within the brain (reviewed in Battaini et al 2001). It has been shown in previous studies that PKC activity involving these neuronal isoforms is required for the rewarding effects of drugs of abuse (Aujla and Beninger 2003; Harlan *et al.* 2004).

The benzophenanthridine alkaloid chelerythrine is a potent, selective antagonist of the Ca++/phospholopid-dependent protein kinase (PKC) from the mouse brain (Herbert et al 1990). Previous studies have used a one trial peck-avoidance task in chicks to show that chelerythrine induced PKC inhibition blocks the formation of memory for these tasks (Serrano et al 1995). Chelerythrine has also been previously shown to block CPP when administered selectively in the nucleus accumbens (Li et al 2011) and ventral tegmental area (VTA) (Chen et al 2012), but sensitization has not been examined. On a molecular level, chelerythrine has been found to block the glutamatergic inputs into the VTA that contribute to dopamine neuronal activation related to reward and response-initiating effects in drug abuse,

demonstrating the PKC pathway is required for these effects. (Velásquez-Martinez MC et al 2012)

As much as chelerythrine has been used, it has never been examined when given continuously and intracerebroventricularly (i.c.v). This study examines the effects of PKC inhibition mediated through chelerythrine (i.c.v) on CPP and sensitization. We hypothesize that chelerythrine will induce PKC inhibition leading to a negation of sensitization but will have no effect on place preference.

II.

Materials and Methods

Animals

17 inbred C57BL6/J mice (11 male 6 female) obtained from Jackson Laboratories (West Sacramento, CA) were used for this study. Mice were weaned at three weeks. The mice all had unlimited food and water, and were housed in a cage with 1 to 4 other mice. The lab vivarium was kept at a 14:10 light dark cycle and all of the testing performed in this study was done during the light phase. Mice were at least 15 weeks of age at the time of testing. The UCSD IACUC approved all animal care and testing procedures and all procedures were in accordance with the NRC Guide for the Care and Use of Laboratory Animals.

Surgery

Before surgery the mice were anesthetized with 2-4 % volume isoflurane and mounted in a stereotaxic apparatus (Benchmark, myneurolab.com). A single hole was drilled in the skull for infusion into the third ventricle (AP: −0.5 mm; ML: 0 mm, DV: −3 mm, relative to bregma; Franklin & Paxinos). A continuous inhibition of PKC (using chelerythrine) was utilized through osmotic mini pumps(100 uL) (Alzet model 1002, Durect Corporation, Cupertino, CA) and PE60 tubing were implanted subcutaneously and connected to 30-ga stainless steel cannulas (Alzet, Brain infusion kit 3). Pumps were also filled with aCSF (100 uL) and connected to PE60 tubing containing aCSF. Fluid was delivered at a rate 0.25 uL/hr (manufacturer specifications, Alzet) and pumps were filled 16 h prior to surgery. Following surgery animals received an injection of buprenorphine (s.c.) and were allowed to recover for 3 days.

Conditioned Place Preference

Apparatus

In the windowless place preference room there are 8 individual chambers that measure 44 cm x 44 cm x 31 cm. A black wall bisects these chambers with a removable insert creating two sides, each distinctive with regard to tactile, odor, and visual cues. One side of the chamber is made of white acrylic walls which contain stickers and has a floor made of metal bars. The other side is made of clear acrylic walls and has a mesh wire floor. The left side of the chambers were cleaned and scented with seven percent isopropyl while the right side of chambers was cleaned with water. The chambers were counterbalanced. Two 100-watt bulbs lit the room and a speaker continuously playing white noise (65 dB) provided background noise.

Place Preference

The mice were habituated to the chambers in two training sessions across two days, 30 minutes each session. Mice then underwent 5 cocaine-context pairings. All mice first received an initial injection of saline and were restricted to the saline side of the chambers for 15 minutes. After, the mice were given a cocaine injection and placed in the drug paired side of the chambers for 15 minutes. After 5 days of training, the mice were given a 24 hour rest period and a place preference test was conducted. During testing, the mice were not given any injections and the insert bisecting the chamber was removed allowing the mice to move freely to both sides of the chamber. The test was conducted for 15 minutes and Activity monitor software (Med-Associates, VT) was used to measure distance traveled and time spent on both sides.

Sensitization

24 hours later mice were given a sensitization test. All mice received an injection of cocaine and mice were restricted to the drug-paired side of the chamber. The sensitization test lasted for 45 minutes and Activity monitor software (Med-Associates, VT) was used to measure the distance traveled.

Drugs

Cocaine (15 mg/kg) and saline were administered intraperiotneally (i.p.). Chelerythrine chloride (Enzo Life Sciences, Farmingdale, NY) was dissolved in PBS to achieve a final concentration of 10 nmol/µL. This dose has previously been demonstrated to effectively inhibit PKC and behavioral memory (Serrano et al., 2008). Buprenorphine hydrochloride (Buprenex®, Reckitt Benckiser Healthcare, Berkshire, UK) was dissolved in 0.9% sodium chloride to achieve a concentration of 0.005 mg/mL. Each mouse was given 0.1 mL subcutaneously (s.c.). Cocaine HCl (Sigma-Aldrich, St. Louis, MO) was administered intraperitoneally (i.p.) at a volume of 10 ml/kg and given at a dose of 15 mg/kg and was dissolved in saline. Mice were randomly assigned to one of two groups: experimental chelerythrine group (n = 9), and control aCSF group (n = 8).

Statistical Analysis

The effect of chelerythrine on the development of sensitization, measured as distance traveled (cm), was assessed using repeated measures (session) ANOVA. Additionally, sensitization measured as the difference in distance traveled during the challenge test and the acute response was assessed using repeated measures (minute) ANOVA. A univariate ANOVA was used to assess differences between

groups on the place preference test. The level of significance was set at $p \leq 0.05$. Results stated as “not significant” or “did not differ” indicate p values > 0.05.

III.

Results

Training

Locomotor activity for session one of training is shown in figure 2. The acute response to cocaine between groups did not differ. Figure 3 depicts average locomotor activity across the 5 sessions of training. There was a significant session by group interaction *(F[4,60] = 3.592, p = .011).

Place Preference

Figure 4 depicts place preference, measured as the difference between time spent on the paired and unpaired sides. Univariate analysis between subjects showed no significant effect between the aCSF and chelerythrine groups. A value greater than zero meant more time spent on the drug side, while a value less than zero meant more time spent on the saline side.

Sensitization

Figure 5 shows the locomotor sensitization (challenge) test. There were no significant between-group differences during the challenge test. Sensitization, measured as the difference in locomotor activity between the challenge test and the acute response, is depicted in Figure 6 A. This difference score accounts for baseline differences between animals. There was a main effect of group *(F[1,15] = 11.102, p = .005). The chelerythrine group had a decreased locomotor activity in comparison to the aCSF group. Figure 6 B shows the average sensitization for each group. Locomotor activity of the chelerythrine group was significantly lower than the aCSF group at *(F[1,15] = 11.102, p = .005).

IV.

Discussion

This investigation examined the role of PKC in the brain in relation to drug reward memories. Although it was shown in previous studies that PKC activity is required for the rewarding effects of drugs of abuse (Aujla and Beninger 2003; Harlan *et al.* 2004), this experimental finding has never been done using chelerythrine i.c.v with continuous administration when looking at sensitization. The results from this study show that in the brain, PKC is required for cocaine induced sensitization in mice. This demonstrates that PKC is a vital protein involved in the mechanism of transition from casual to compulsive drug use in addicts. The results from this study also showed that PKC is not a protein involved in the mechanism of context memory formation which occurs during conditioned place preference. it is likely not involved in the drug seeking behavior which underlies addiction.

These findings show a significant group by session interaction with regards to locomotor activity. This means that over the 5 sessions, the mice given cocaine had a greater increase in the amount locomotor activity per session in comparison to the mice given saline. This finding is consistent with the results of previous studies in which repeated administration of cocaine to mice increased their ambulation as much as four-fold over the response to the first injection (Shuster et al 1977).

The finding that chelerythrine does block sensitization in mice is consistent with a previous finding in our lab where ZIP,a PKMζ (PKC isoform) inhibitor, also blocked sensitization (Unpublished data) While ZIP only blocks the PKMζ isoform of PKC and chelerythrine blocks PKC less selectively(Yao et al 2012), the conclusion that PKC mediates sensitization can be reached.

The finding that chelerythrine does not block place preference is also consistent with other findings in our lab that use ZIP as the PKMζ inhibitor (Unpublished data). This means that chelerythrine may not block the formation of the context memory created in place preference. This result was inconsistent with other results from Li et al 2011 which showed that place preference was blocked when ZIP was injected in the nucleus accumbens core. This discrepancy may have been due to the fact that by giving directly to the accumbens they were giving a more concentrated dose into a specific area, thus getting the concentration needed to block place preference. Or the i.c.v injections of chelerythrine did not diffuse all the way into the nucleus accumbens, and thus were not able to inhibit PKC in the accumbens. This confound was also seen when drugs are injected intracranially by Routtenberg et al (1972) and Hoffman et al (1992). They found that drugs typically diffuse up the cannula shaft and reach brain areas dorsal to the intended injected site (Routtenberg, 1972; Wise and Hoffman, 1992). Thus, a question for future research is where exactly the chelerythrine was able to diffuse to, and where it was able to act upon in the brain. This question would be answered by a western blot of Adducin, a major PKC substrate (Matsuoka et al. 1996, 1998). Adducin is a ubiquitously expressed calmodulin-binding protein (Gardner and Bennett, 1986; Bennett et al., 1988) and substrate for protein kinase C (Palfrey and Waseem, 1985; Cohen and Foley,1986; Ling et al., 1986; Kaiser et al., 1989), making it an ideal protein to analyze. Previous studies looking for PKC inhibition have also looked at western blots for Adducin because of its involvement with PKC (Matsuoka et al. 1996, 1998), and thus confirming the down regulation of Adducin would confirm the down regulation of PKC. Another future study would be to inject chelerythrine just before training instead of

continuously throughout the experiment and examine any changes to the negation of sensitization and or place preference.

Other future studies will implant the cannula into more specific regions of the brain such as the ventral tegmental area and hippocampus to determine if inhibiting PKC specifically and locally in these areas has any effect on blocking sensitization and or place preference. These localized studies would coalesce with the western blot studies to determine where the effect of PKC inhibition by chelerythrine has most potency.

This study examined the effects of chelerythrine induced PKC inhibition in the brain when given continuously, i.c.v. It was found that PKC activity underlies cocaine-induced sensitization and thus PKC may be necessary for the transition from casual to compulsive use in addicts modeled by sensitization. However, this paradigm did not inhibit place preference. This study demonstrated that PKC activity is involved in the memories of cues that cause relapse to drug addicted populations and could be used as a potential therapeutic target.

Appendix

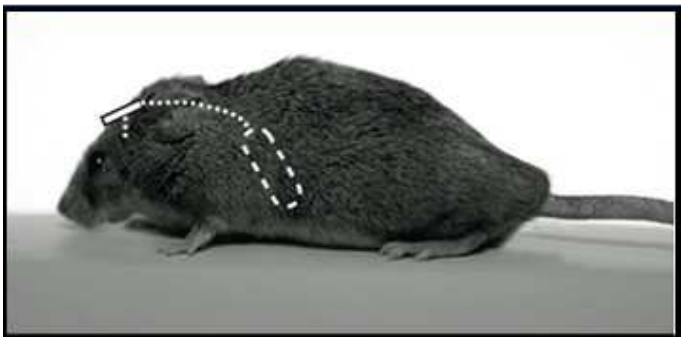

Figure 1. . An image of a mouse post surgery with the osmotic mini pump (Alzet model 1002, Durect Corporation, Cupertino, CA) and PE60 tubing implanted subcutaneously and connected to 30-ga stainless steel cannulas (Alzet, Brain infusion kit 3).

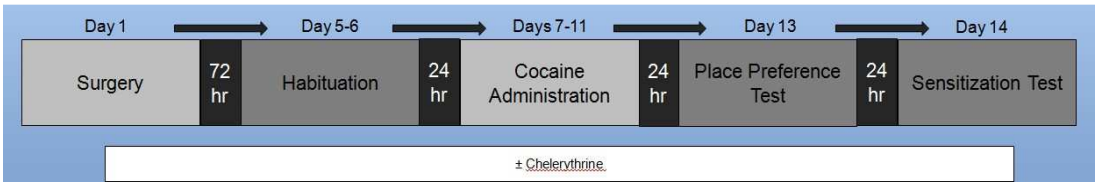

Figure 2. Timeline of the experimental procedure. On day 1 surgery was done and a cannula was placed into the third ventricle and an Alzet micro osmotic pump and tubing was placed subcutaneously which were then subsequently attached. Mice were then given 3 days of rest before habituation began. After two habituation sessions, 5 sessions of training began. After 5 days of training, the mice were given a 24 hour rest period and then a place preference test was conducted, during which time all animals were off drug. Mice were then given a 45 minute sensitization test twenty four hours later. All mice received cocaine.

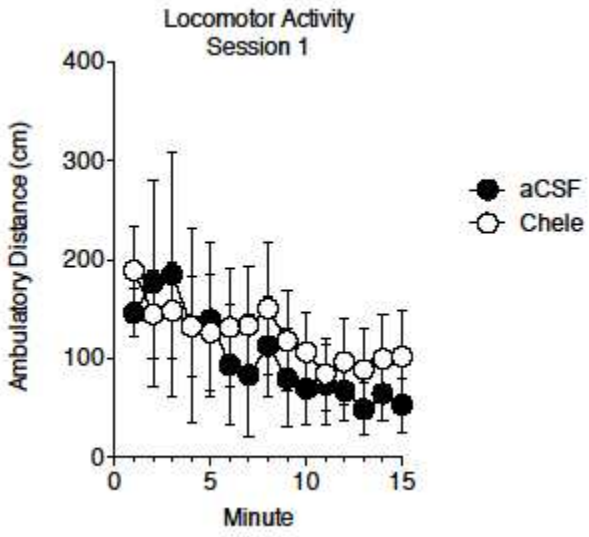

Figure 3. Locomotor activity for session one of training. Mice were given an injection of Cocaine (15 mg/kg) and placed in the drug paired side of the chamber. Their ambulation was assessed and this figure displays the animals' acute response to the cocaine. There was no significant main effect or minute by group interaction.

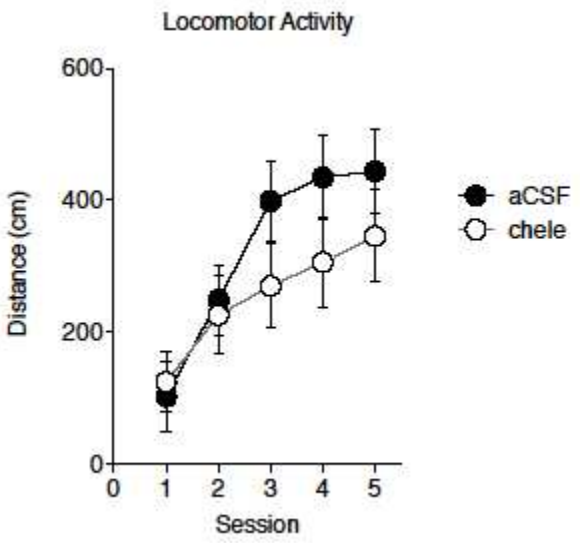

Figure 4. Locomotor activity over the 5 sessions of training. Both groups received a 15 mg/kg dose of cocaine and placed in the drug paired side of the chamber for 5 sessions over 5 days. Average locomotor activity was assessed. Repeated measures ANOVA showed a significant session by group interaction *($F[4,60] = 3.592$, $p = .011$).

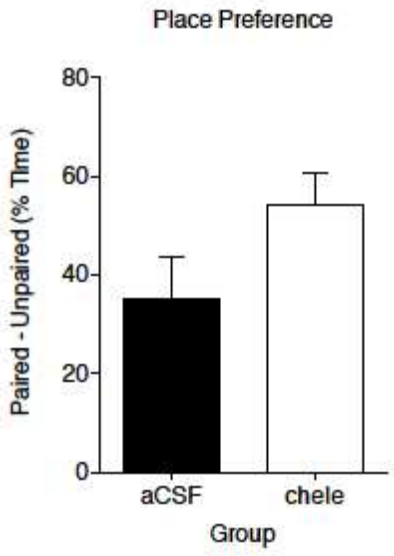

Figure 5. Place Preference. Paired – Unpaired Percent Time Spent. The mice were not given any injections and the insert bisecting the chamber was removed allowing the mice to move freely to both sides of the chamber. Time spent of both the drug and saline side was measured and a univariate analysis between subjects showed no significant effect between aCSF and chelerythrine groups.

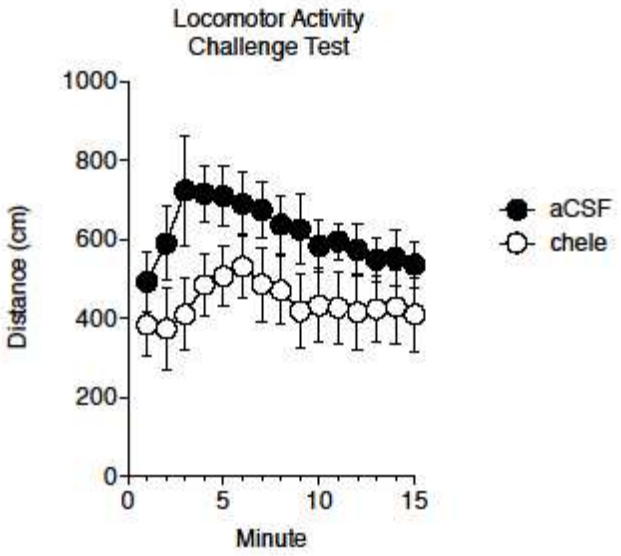

Figure 6. Sensitization (Challenge) Test. 48 hours after training a sensitization test was conducted. All groups were given a 15 mg/kg dose of cocaine, were restricted to the drug-paired side of the chamber, and their ambulatory distance was assessed. No significant interaction and no significant main effect were seen.

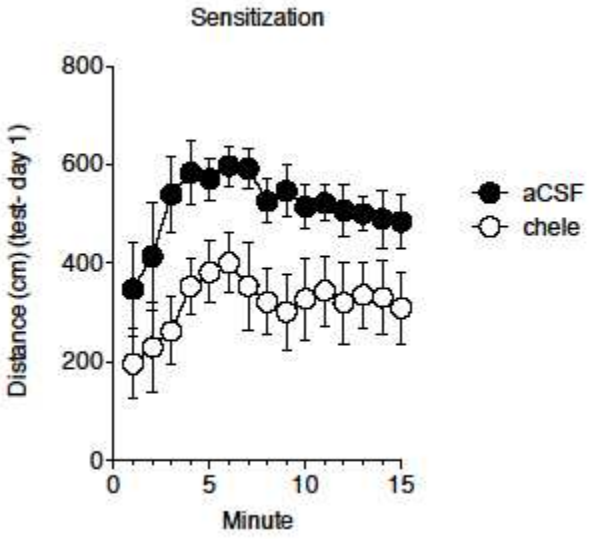

Figure 7. Sensitization. Acute locomotor activity was subtracted from locomotor activity recorded during the challenge test. A significant effect of group *(F[1,15] = 11.102, p = .005) was found showing that the chelerythrine group had a decreased locomotor activity in comparison to the aCSF group.

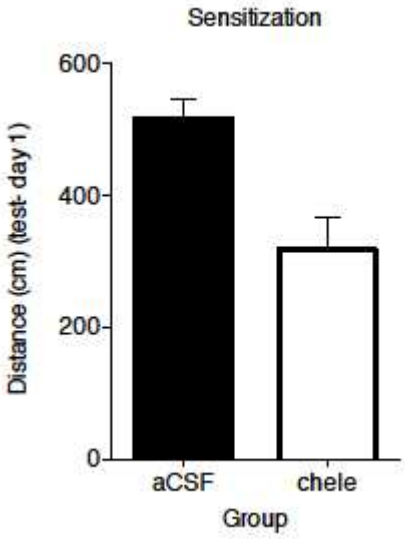

Figure 8. Sensitization (average). A bar graph showing the results of A above. The reduced locomotor activity of the chelerythrine group was found to be significant at *(F[1,15] = 11.102, p = .005).

References

Aujla H. and Beninger R. J. (2003) Intra-accumbens protein kinase C inhibitor NPC 15437 blocks amphetamine-produced conditioned place preference in rats. *Behav. Brain Res. 147, 41–48.*

Battaini F.(2001) Protein kinase C isoforms as therapeutic targets in nervous system disease states. *Pharmacol Res ;44:353–61. [PubMed: 11712865]*

Bennett, V., K. Gardner, and J.P. Steiner. (1988). Brain adducin: a protein kinase C substrate that may mediate site-directed assembly at the spectrin-actin junction. *J. Biol. Chem. 263:5860–5869.*

Brennan A. R., Yuan P., Dickstein D. L., Rocher A. B., Hof P. R., Manji H. and Arnsten A. F. (2009) Protein kinase C activity is associated with prefrontal cortical decline in aging. *Neurobiol. Aging 30, 782–792*.

Ciccocioppo R, Martin-Fardon R, Weiss F (2004) Stimuli associated with a single cocaine experience elicit long-lasting cocaine-seeking. *Nat Neurosci 7:495– 496.*

Cohen, C.M., and S.F. Foley. (1986). Phorbol ester- and Ca11-dependent phosphorylation of human red cell membrane skeletal proteins. *J. Biol. Chem. 261:7701–7709.*

Gardner, K., and V. Bennett. (1986). A new erythrocyte membrane-associated protein with calmodulin binding activity. *J. Biol. Chem. 261:1339–1348.*

Grimm JW, Hope BT, Wise RA, Shaham Y (2001) Incubation of cocaine craving after withdrawal. *Nature 412:141–142*

Harlan R. E., Kailas S. R., Tagoe C. E. and Garcia M. M. (2004) Morphine actions in the rat forebrain: role of protein kinase C. *Brain Res. Bull. 62, 285–295.*

Herbert JM, Augereau JM, Gleye J, Maffrand JP.(1990) Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem Biophys Res Commun. Nov 15;172(3):993-9.*

Hinson RE, Poulos CX. (1981) Sensitization to the behavioral effects of cocaine: modification by Pavlovian conditioning. *Pharmacol Biochem Behav. Oct;15(4):559-62*

Ho SY, Chen CH, Liu TH, Chang HF, Liou JC. (2012) Protein kinase mζ is necessary for cocaine-induced synaptic potentiation in the ventral tegmental area. *Biol Psychiatry. Apr 15;71(8):706-13. Epub 2011 Dec 9.*

Hunt WA, Barnett LW, Branch LG (1971) Relapse rates in addiction programs. *J Clin Psychol 27:455– 456.*

Kaiser, H.W., E. O'Keefe, and V. Bennett. (1989). Adducin: Ca21-dependent association with sites of cell–cell contact. *J. Cell Biol. 109:557–569.*

Ling, E., K. Gardner, and V. Bennett. (1986) Protein kinase C phosphorylates a recently identified membrane skeleton-associated calmodulin-binding protein in human erythrocytes. *J. Biol. Chem. 261:13875–13878*

Yan-qin Li,1 Yan-xue Xue,1 Ying-ying He,1 Fang-qiong Li,1 Li-fen Xue,1 Chun-mei Xu,1 Todd Charlton Sacktor,2,3 Yavin Shaham,4 and Lin Lu1 (2011) Behavioral/Systems/Cognitive Inhibition of PKM! in Nucleus Accumbens Core Abolishes Long-Term Drug Reward Memory National Institute on Drug Dependence, *The Journal of Neuroscience, April 6 • 31(14):5436 –5446*

Matsuoka Y., Li X. and Bennett V. (1998) Adducin is an in vivo substrate for protein kinase C: phosphorylation in the MARCKS-related domain inhibits activity in promoting spectrin-actin complexes and occurs in many cells, including dendritic spines of neurons. *J. Cell Biol. 142, 485–497.*

Mucha RF, van der Kooy D, O'Shaughnessy M, Bucenieks P (1982) Drug reinforcement studied by the use of place conditioning in rat. *Brain Res 243:91–105*

O'Brien CP, Ehrman RN, Ternes JW (1986) Classical conditioning in human opioid dependence. *In: Behavioral analysis of drug dependence Goldberg S, Stolerman I, eds, pp 329–356. Orlando: Academic.*

Palfrey, H., and A. Waseem. (1985). Protein kinase C in the human erythrocyte. Translocation to the plasma membrane and phosphorylation of bands 4.1 and 4.9 and other membrane proteins. *J. Biol. Chem. 260:16021–16029.*

Robinson, T. E. B., J.B. (1986). Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Res, 11, 157-198.*

Routtenberg A (1972) Intracranial chemical injection and behavior: a critical review. *Behav Biol 7:601– 641.*

Segal, D. S., Kuczenski, R. (1994). Behavioral Pharmacology of Amphetamine. In A. K. S. Cho, D.S. (Ed.), *Amphetamine and its analogs: psychopharmacology, toxicology and abuse (pp. 115-150). San Diego: Academic.*

Rodriguez WA, Pope B, Bennett EL. (1995) Protein kinase C inhibitor chelerythrine disrupts memory formation in chicks. Serrano PA. Rosenzweig MR *Behav Neurosci. Apr;109(2):278-84*

Shuster L, Yu G, Bates A. (1977) Sensitization to cocaine stimulation in mice. *Psychopharmacology (Berl). Apr 29;52(2):185-90.*

Stewart J (1992) Neurobiology of conditioning to drug abuse*. Ann N Y Acad Sci 654:335–346.*

Velásquez-Martinez MC, Vázquez-Torres R, Jiménez-Rivera CA.(2012) Activation of alpha1-adrenoceptors enhances glutamate release onto ventral tegmental area dopamine cells. *Neuroscience. Aug 2;216:18-30. Epub 2012 Apr 24.*

Wikler A (1973) Dynamics of drug dependence, implication of a conditioning theory for research and treatment. *Arch Gen Psychiatry 28:611– 616.*

Wise RA, Hoffman DC (1992) Localization of drug reward mechanisms by intracranial injections. *Synapse 10:247–263.*

Yamanaka, Y., Yamamoto, T., Egashira, T. (1983). Methamphetamine-induced behavioral effects and releases of brain catecholamines and brain concentrations of methamphetamine in mice. *Jpn J Pharmacol, 33(1), 33-40.*

Yao Y, Shao C, Jothianandan D, Tcherepanov A, Shouval H, Sacktor TC. (2012). Matching biochemical and functional efficacies confirm ZIP as a potent competitive inhibitor of PKMζ in neurons. *Neuropharmacology. Jul 27.*

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