Regulation of Alpha Synuclein Following Chronic Methamphetamine Administration in Guinea Pigs: Correlation with Memory and Synaptic Plasticity

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REGULATION OF ALPHA SYNUCLEIN FOLLOWING CHRONIC METHAMPHETAMINE ADMINISTRATION IN GUINEA PIGS: CORRELATION WITH MEMORY AND SYNAPTIC PLASTICITY

A Thesis in Biomedical Sciences

By

Adnouse Blanc

Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Biomedical Sciences

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Abstract

Methamphetamine (METH) is a highly addictive drug of abuse that has a severe impact on neuronal changes in the brain including modulations of plasticity, cognitive dysfunction, as well as memory impairment. These changes can be seen as modifications in the expression of biochemical markers associated with synaptic plasticity. One such marker associated with memory impairment is alpha synuclein (α-syn). Alteration of α-syn expression has been linked to memory impairment in patients with Alzheimer’s disease (AD) and Parkinson’s disease (PD). Here we assess the effect of chronic METH treatment in correlation to cognitive functions.

Twenty-nine guinea pigs (male, 150-250 g) were subcutaneously inserted with ALZET osmotic mini-pumps to deliver either a) saline (24 μl/day), b) METH (10 mg/kg) per day for 7 days or c) Post METH washout (10mg/kg). On Day–7, the Novel Object Recognition test (NOR) was used to assess memory recall. Electrophysiological techniques were used to assess synaptic plasticity, in the hippocampus CA1 subfield, as it relates to learning and memory (n=6). Western blots were used to evaluate the expression of α-syn in the hippocampus.

Saline treated animals (n=7) and the Post METH washout (n=12) showed a preference for the novel object as compared to the METH treated animals (n=10) that had a preference for the familiar object even after 7 days post treatment (n=12). Molecular assays showed a down regulation of endogenous alpha synuclein protein levels in the hippocampus between subgroups. Furthermore, long-term potentiation (a cellular correlate of memory) was maintained in all subgroups.
The results from this study warrant the conclusion, that METH affects some of the same mechanisms underlining memory function and the drug-induced activation lead to the memory impairments observed in METH addicts and the role of alpha synuclein in the memory function still remains unclear.
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Literature review

1.1 Methamphetamine

The abuse of Methamphetamine (METH) in the United States has increased markedly in the past 15 years (Cretzmeyer et al., 2003; Rawson et al., 2002; Marshall et al., 2007). Due to its inexpensive production and low cost, METH is the most synthesized illegal drug in the USA with an increasing pattern of use in pre-teens and adolescents (Cretzmeyer et al., 2003) See Figure 1 for molecular structure of METH. Second to Cannibas, METH is the most popular drug of recreational use in the United States. It is a synthetic derivative of amphetamine, highly addictive, and chronic use has been linked to several neurocognitive impairments (Ares-Santos, et al., 2011).

METH can enter the central nervous system (CNS) passively or through the dopamine transporters (DAT) and acts on the CNS through a non-exocytotic mechanism, causing the release of monoamine neurotransmitters such as dopamine, norepinephrine and serotonin (Makisumi et al., 1998). METH has the ability to (i) redistribute catecholamines from the synaptic vesicles to the cytosol, (ii) reverse the transport of the neurotransmitters through plasma membrane transporters (Khoshbouei et al., 2003), and (iii) inhibit the activity of monoamine oxidase (MAO), an enzyme that degrades amine neurotransmitters such as dopamine, serotonin, and norepinephrine (Barr et al., 2006; Mantle et al., 1976).

Typically, individuals who chronically abuse METH display signs of acute toxicity, neurological damage, hypertension, aggression, impaired judgments, seizures, anxiety, depression and altered behavior and cognitive function (Albertson et al., 1999;
Barr et al., 2006; Murray 1998). Neuroimages of METH abusers show many morphological changes that occur with sustained usage. These changes include persistent decreases in the levels of dopamine transporters (DAT) in the orbitofrontal cortex, dorsolateral prefrontal cortex, and the caudate-putamen (Iyo et al., 2004; McCann et al., 1998; Sekine et al., 2003; Volkow et al., 2001), and a decrease in the density of serotonin transporters (5-HTT) in the midbrain, caudate-putamen, hypothalamus, thalamus, and the orbitofrontal, temporal, and cingulate cortices (Sekine et al., 2006; Aron and Paulus, 2007; Chang et al., 2007). As demonstrated by Thompson et al., (2008), METH exposure can result in extensive neuronal damages that can be seen distinctly in neuro imaging techniques in animals in vivo. For example, magnetic resonance imaging (MRI) has shown morphological changes in the brain that consist of a loss of gray matter in the cingulate, limbic and paralimbic cortices, along with significant shrinkage of the hippocampi and hypertrophy of white matter.

METH has relatively high lipid solubility, allowing for more rapid transfer of the drug across the blood–brain barrier especially in the hippocampus (Martins et al., 2011). The increase of the drug in the brain can compromise the permeability of the blood brain barrier in the hippocampus within 24 hours after injection in young adult mice (Martins et al., 2011). The lipophilic ability of METH is likely attributed to its 10-12 hour plasma half-life in humans (Schepers et al., 2003; Martins et al., 2011). Furthermore, repeated exposure of moderate-to-high doses of METH produces blood-brain barrier disturbances
Figure 1. The structure of METH. Adapted from http://www.sigmaaldrich.com/catalog/product/sigma/m8750
that induce neuronal damages, myelin degeneration, reactive astrocytosis in the parietal and occipital cortices of the brain (Sharma et al., 2009) and long-term damage to presynaptic dopaminergic and serotonergic terminals (Wagner et al., 1980; Ricaurte et al., 1982; Wilson et al., 2003; McCann et al., 1998).

While the cellular and neurochemical repercussions of various METH dosing regimens have been investigated extensively, less is known concerning the cognitive function of humans and animals following chronic METH administrations. Neuropsychological studies have detected deficits in attention, working memory, and decision making with active METH use (Gonzalez et al., 2007; Verdejo-Garcia et al., 2006; Woods et al., 2005). Additionally, withdrawal from METH can produce irritability, fatigue, impaired social functioning, and intense craving for the drug (Brecht et al., 2004; Darke et al., 2008; Homer et al., 2008; Sekine et al., 2006; Zweben et al., 2004). Even after 3 months of abstinence, recovering METH abusers score worse on word recognition and tests of episodic memory than individuals who do not use METH (Simon et al., 2004).

Drugs of abuse such as METH can utilize synaptic circuits in the mesolimbic/mesocortical or the nigrostriatal pathway to induce abnormal drug reward systems and extreme addictions (Kalivas and Volkow, 2005; Jones & Bonci, 2005, Hyman et al. 2006) as indicated in Figure 2. The mesolimbic dopamine pathway contains neurons that originate in the A10 region of the ventral tegmental area (VTA) of the midbrain and project to the nucleus accumbens and other structures of the basal forbrain, including the hippocampus and the caudate-putamen (Bjorklund and Lindvall, 1975). Wise and Bozarth postulated that psychostimulants use the
mesolimbic/mesocortical pathways to produce euphoric effects in humans (1985). The nigrostriatal dopamine pathway originates in the substantia nigra pars compacta and projects into the striatum (Bjorklund and Lindvall, 1975). The nigrostriatal pathway plays a primary role in movement initiation and control. The functional significance of this pathway is best illustrated in Parkinson’s disease (PD) that results in rigidity, bradykinesia and some form of memory loss (Bannon et al., 2005).

Taken together, METH use is shown to be associated with neurocognitive impairments that include poor performance on measures of attention/information processing speed, learning and memory, and frontal lobe functioning (Kalechstein and Newton 2003; Quinton and Yamamoto 2006). Memory tasks are the most pronounced deficits in METH users. These memory deficits have been demonstrated on tests of verbal (Hoffman et al., 2006), working (Gonzalez et al., 2007), prospective (Rendell et al., 2009), and episodic memory (Simon et al., 2004). Even after METH use has stopped, 40% of the individuals still experience neurocognitive impairments (Cherner et al., 2010). Clinically, current and abstinent METH users show impairments in tests of verbal memory, manipulation of information, and motor performance (Volkow et al., 2001; Simon et al., 2002; Thompson et al., 2004). These observations further support the hypothesis that METH has an effect on learning and memory.
Figure 2. Modified diagram of the mesolimbic/nigrostriatal pathways. METH can use these pathways to induce neuronal damage to the limbic system. Adapted from www.elsevierimages.com/image/33387.
1.2 The Hippocampus

As part of the limbic system, the hippocampus is located on the medial surface of the temporal lobe and has major functions in learning and memory (Kuno, 1995). The hippocampus is divided into four sections that include the dentate gyrus, the hippocampus proper, the subiculum, and the entorhinal cortex. Within the different sections of the hippocampus, there are several molecular layers that include (1) an external plexiform layer, which consist of axons of pyramidal cells that project outside the hippocampus and hippocampal afferent fibers from the entorhinal cortex; (2) stratum oriens with basal dendrite and basket cells (3), a pyramidal cell layer that contains the pyramidal cells of the hippocampus, and (4) the stratum radiatum and stratum lacunosum moleculare (Arbib et al., 1998).

The entorhinal cortex (EC) is the major source of projections into the hippocampus via the perforant pathway. Two-thirds of the cortical input to the entorhinal cortex originates in the perirhinal and parahippocampal cortices, which in turn receive projections from areas in the frontal temporal, and parietal lobes of the brain (Squire and Zora-Morgan, 1991) As seen in Figure 3, the EC projects to the dentate gyrus (DG) and the CA3 through the perforant path (pp) (Witter et al., 1988, 2007). In turn, the dentate gyrus (DG) projects to the CA3 through the mossy fibers (mf). The CA3 projects to the CA1 through the Schaffer Collateral (SC) and the CA1 projects outside the hippocampus back to the of the entorhinal cortex in a loop. Also, the CA1 can receive direct information from the EC and the CA3.

The hippocampus and adjacent structures in the medial temporal lobe memory
system function as storage for simple memory (Marr, 1971). The hippocampus is crucial for rapid acquisition of new information about facts and events, which is available as conscious recollection (Squire and Zola, 1996).
Structure of the Hippocampus

Figure 3. The structure of the hippocampus. The EC projects to the dentate gyrus (DG) and the CA3 through the Perforant Path. In turn, the DG projects to the CA3 through the mossy fiber (mf) which projects to the CA1 through the Schaffer Collateral (sc). The CA1 projects back to the EC. Adapted from en.wikipedia.org
1.3 Memory

Studies of memory have led to a consensus that memory is not a monolithic, but rather is supported by multiple brain systems that differ in terms of the types of memory they mediate. The Multiple Memory Systems Theory states that there are specific circuits that serve specific classes of learning and memory problems (Squire and Zola-Morgan, 1991). This theory was originally derived largely in part from patterns of impaired and spared learning abilities following damages to the mammalian hippocampal system (Cohen and Squire, 1980; Hirsh, 1974; Saunders et al., 1984; Squire, 1992).

According to McGaugh et al. (2000), there are three main memory systems: working memory, short-term memory, and long-term memory. See Figure 4 for a schematic of different memory groups. Working memory system occurs immediately while an event is presently occurring and only last for a few seconds to a few minutes (McGaugh et al., 1966; Izquerido et al., 1999; Gold et al., 1975; Goldman-Rakic et al., 1992). The Izquerido et al. (1999), study demonstrate that working memory does not retain an archive of memories, but rather moment to moment events that may or may not relate to events already stored in the brain. Working memory serves simultaneous information, storage, and processing functions (Baddeley, 2000). Working memory impairments are commonly found in older adults who often have difficulty performing dual tasks that require both storage and processing (Salthouse, 1996);

Short-term memory, on the other hand, is the ability to acquire new information and retain it for periods of time ranging from a few seconds or a few minutes and lasts for several hours (Tulving et al., 1997; Gold et al., 1975). Short term memory requires
Memory Systems

Figure 4. Types of human memories. Adapted from http://www.human-memory.net.
rehearsal or repetition of the information and sorting or categorization of that information into systematic chunks. The sorting process is usually called coding or chunking (Gold et al., 1975). Short-term memory is thought to be translated into long-term memory via permanent modification of proteins or by the synthesis of new proteins (Costa-Mattioli, 2008; Moncada and Viola, 2007). Long-term memory involves the retention of new information and lasts from hours to even a lifetime (Levitian and Kaczmarek, 1991).

Long-term memory can further be divided into declarative and non-declarative memory (Levitian and Kaczmarek, 1991). Declarative memory refers to the capacity to consciously remember the past and depends on the integrity of the medial temporal lobe structures (Squire and Clark, 2007). Declarative memory or explicit memory is the conscious recollection of specific facts and events (Squire and Zola-Morgan 1996). Explicit memory, a subgroup of declarative memory, is explicitly located in the past and is accompanied by the feeling of remembering when subjects can retrieve the correct item (Ennaceur, 2010). This type of long-term memory encourages recognition memory. In preclinical rodent models, components of episodic memory can be evaluated by an object recognition task to assess recognition memory (Ennaceur, 2010). Non-declarative memory or implicit memory is the non-conscious learning capacities that include memory for skills and procedures (Squire and Zola-Morgan 1996). Non-declarative memories are procedural skills that are learned through practice such as riding a bike (Willingham, 1998). It is often slow, learned in segments, and requires immediate and constant feedback along with repetition (Willingham, 1998). Consolidation of long-term memory is slow, passive, and relatively permanent (Wolfman et al., 1994). A neuronal
mechanism to assess learning and memory is synaptic plasticity. It is a process that is actively occurring as memory moves from short term to long term memory.
1.4 Synaptic Plasticity

Learning and memory require persistent changes in neuronal circuits (Nestler et al., 2004). These changes, according to the Hebbian theory, occur when the connections between coactive cells change and thereby store a record of the event (Cooper, 2005). This is due to the increase in synaptic efficiency that arises from the cell’s repeated and persistent stimulation of the postsynaptic cell to induce what is known as synaptic plasticity. According to Hebb, if the presynaptic and the postsynaptic elements are coactive and calcium influx is sufficient, then the strength of the synapse increases (Cooper, 2005). The ability of the brain to adapt and respond to changes in the environment is referred to as neuroplasticity (Nestler et al., 2004). This plasticity is important for learning and memory as it is the underlying mechanism for memory formation (Nestler et al., 2004; Jones & Bonci, 2005). Thus, learning and memory require neuronal mechanisms that allow rapid, persistent changes to brain circuits to account for plasticity (Shapiro, 2001). The circuits that are located within the hippocampus are remarkably plastic, and this plasticity is mediated in part through changes in synaptic strength.

Synaptic plasticity can be defined by two parameters: (1) the amount of transmitter released from the presynaptic terminals and (2) the size of the postsynaptic response produced per unit amount of transmitter (Kandel, 2009). Plasticity occurs when there are changes in one or both of the parameters. It can be divided into short–term (minutes to hours) and long-term (days to years) (Kandel and Schwartz, 1982). Long-term synaptic plasticity is a form of plasticity that is relevant in the efficiency of learning
and memory in the brain because it also requires changes in synaptic structures and gene expression (Malenka and Bear, 2004; Kandel, 2009). The most well-known and studied mechanism of neural plasticity is long-term potentiation (LTP) or long-term depression (LTD) as these long-lasting synaptic changes are widely believed to be the cause of many forms of long lasting memory changes (Bliss and Lomo, 1973; Kandel, 2009).

LTP is defined as an activity-dependent enhancement of synaptic strength and is the general cellular model of learning-induced plasticity in the hippocampus (Bliss et al., 1973; Clarke et al., 2010). The term LTP was coined by Bliss and Lomo in 1973, in an experiment when a brief tetanic stimulation (10-100 hertz for 3-20 sec) was applied to the perforant path presynaptic fibers in the dentate gyrus of a rabbit. The postsynaptic response was recorded from the granular cells of the hippocampal formation and lasted for more than 3 days (Bliss and Lomo 1973). The results suggested that two independent mechanisms were responsible for LTP: an increase in the efficiency of synaptic transmission at the perforant path synapses and an increase in the excitability of the granule cell population (Bliss and Lomo, 1973).

Classic LTP has been characterized by two phases, (1) early-LTP that is initiated in the absence of protein synthesis (1-2 hrs) and (2) a late-LTP that requires the synthesis of new proteins and lasts longer than 3 hours (Huang and Kandel., 1998). Early-LTP is typically initiated with a single train of high-frequency stimuli (100 Hz, 1 sec) and starts immediately after post-tetanic stimulation (Huang and Kandel., 1998; Kandel, 2009). Once early-LTP is initiated, it is maintained by the activation of NMDA receptors which leads to the influx of calcium and leads to the activation of secondary messengers (Malenka et al., 1988; O’Dell and Kandel et al., 1991). It has been suggested that early-
LTP may involve temporary modifications of existing protein (Malenka et al., 1988; Huang and Kandel 1998,). Late-LTP is typically initiated with three trains of high-frequency stimuli (100 Hz, 1 sec, at 60 sec intervals) and typically starts three hours after post-tetanic stimulation (O’Dell and Kandel et al., 1991; Pittenger et al., 2006). Late-LTP also requires the activation of the N-Methyl-D-aspartate (NMDA) receptors, voltage dependent calcium channels, and may require protein synthesis (Raymond, 2007; Nguyen et al.; 2003).

LTP can also be measured by changes in plasticity as represented by ongoing molecular events in the cell. The major excitatory transmitter substance in plasticity is NMDA (Kuno, 1995). According to Shapiro NMDA receptors are crucial for inducing these plastic changes, and blocking these receptors reduces plasticity and impairs learning in tasks that require the hippocampus (2001). Modifications, such as the introduction of a drug or a NMDA antagonist can disrupt signaling mechanisms downstream of the NMDA receptor, leading to the prevention of LTP induction and to the impairment of hippocampus-dependent learning (Kuno, 1995).

In the hippocampus, NMDA receptors are commonly implicated in activating nuclear gene transcription (Worley et al., 1993; Ghosh and Greenberg, 1995; Finkbeiner and Greenberg, 1998). As indicated in Figure 5, once NMDA receptors are activated by neurotransmitters, a signal is generated to secondary messengers to activate also. The increase in second-messenger levels that occurs activates different protein kinases, such as protein kinase A (Shaywitz and Greenberg, 1999) and calcium- and calmodulin-dependent kinase IV (Ginty 1997; Shaywitz and Greenberg, 1999; Wu et al., 2001). The
Figure 5. Summary of the overall biochemical cascade by which ligands such as METH can alter gene transcription in the nervous system. Adapted from: www.chemistry.emory.edu
nuclear targets for the activated kinases include many things, such as regulatory transcription factors (RTFs) like the cAMP response element binding protein (CREB). CREB binds to a cAMP response element (CRE) in many gene promoters including growth factors, enzymes, other transcription factors, and structural proteins (Ginty 1997; Lonze and Ginty, 2002). CREB can also be modified in various ways that can lead to changes in its activity or stability (Johannessen et al., 2004). When CREB binds to CRE it becomes “active” due to the phosphorylation that occurs at Ser-133 (Ginty, 1997). Studies have shown that late-phase LTP at the cortical synapses lead to the increase of Ser-133 phosphorylation of CREB and this increases the CREB activity in the hippocampus (Johannessen et al., 2004; Shaywitz and Greenberg, 1999)). This prolonged Ser-133 phosphorylation of CREB is observed when it is activated by upstream modulators of extracellular signal-regulated kinases (ERK) and may play a role in the maintenance of LTP and normal memory (Wu et al., 2001).

Additionally, CREB activity is increased in reward-related areas of the brain following chronic or acute treatment with psychostimulates such as amphetamine and cocaine (Konradi et al., 1994; Hyman et al., 1996;). The increases in CREB activity following chronic drug use suggest involvement in the reward-related drug addictions and the modification of gene expression (Nestler, 2004).

We have reviewed that chronic drug use has severe impacts on memory formation, reactivation, and reconsolidation suggesting that neuronal changes are occurring within the brain (Huang and Kandel, 1998). These changes in long lasting neuronal plasticity have been the accepted model to study memory formation.
1.5 Alpha Synuclein

The synucleins are small soluble presynaptic proteins expressed primarily in the neural tissue (George, 2002). The synuclein family consists of three distinct proteins that have been described only in vertebrates (Spillantini et al., 1995). The alpha and beta synuclein proteins are found in abundance primarily in the brain tissue while the gamma protein is found primarily in the peripheral nervous system and retina (George, 2002). The first synuclein was identified from a purified cholinergic vesicle from the electric organ of the Pacific electric ray *Torpedo californica* where it was found in both the nerve terminals and the nuclear envelope (Maroteaux et al., 1988).

Alpha–synuclein (α-syn) is a small protein of 140 amino acids, 17 kda, that is localized in the cytosol of the presynaptic terminal of neuronal tissues. α-syn is predominately expressed in the hippocampus, neocortex, thalamus, and the substantia nigra (Yoshimoto et al., 1995; Murphy et al. 2000; Barr et al., 2006; Schultz-Schaeffer, 2010). It belongs to a family of loosely folded proteins (Weinreb et al., 1996; Uversky et al., 2001). Although cellular functions have not been determined for any of the synuclein proteins, but mutations in the α-syn gene have been implicated in many neurodegenerative diseases such as Alzheimer’s and Parkinson disease through abnormal accumulation, gene triplication, or misfolding (Polymeropoulous et al., 1997).

Researchers have suggested α-syn may play a role in modulating numerous cellular functions. These roles include (1) modulating vesicular synaptic release (Murphy et al., 2000), (2) cell signaling, (Kurz et al., 2010), (3) neuronal plasticity (Clayton and George, 1998), (4) synaptic function maintenance (Petersen et al., 1999), (5) regulation
of distinct pools of synaptic vesicles in mature neurons (Murphy et al., 2000), (6) inhabitation of phospholipase D activity which is linked to pathways of cell growth, neurotransmitter release and differentiation (Jenco et al., 1998;), (7) defensive role in response to neuronal cell injury (Kholodilov et al., 1999), functions in oxidative stress (Lee et al., 2006;), and (8) synaptic release by promoting SNARE complex assembly (Perez et al., 2002)

One study has suggested that under normal physiological conditions, α-syn exists in an α-helix rich tetramer that is not prone to aggregation (Bartels et al., 2011). However under disease conditions, the protein may misfold into β-sheet-rich conformations and aggregate into fibrils which are the main components of Lewy bodies and Lewy neurites. A classical Lewy body is an eosinophilic cytoplasmic inclusion that consists of a dense core surrounded by a halo of 10 nm-wide radiating fibrils, the primary structural component of which is α-syn (Bartels et al., 2011; Spillantini et al., 1998;). Under abnormal physiological conditions, disruption of the local alpha helical structure can occur and the β-pleated sheets can be extended to render mutant α-syn that is more prone to self aggregation and abnormal deposition in the brain (Kruger et al., 1998; Polymeropoulous et al., 1997).

Alpha-syn is often associated with neurodegenerative diseases associated with cognitive declines such as Alzheimer disease (AD) and Parkinson’s disease (PD) (Norris et al., 2004). Other studies have shown that an over expression of α-syn or a missense mutation on the gene can lead to rare forms of PD (Polymeropoulous et al., 1997; Spillantini et al., 1998). In the familial form of PD, there is a missense in the α-syn gene, Ala53Thr (Polymeropoulous et al., 1997) or Ala30Pro (Kruger et al., 1998). PD is
clinically classified as a movement disorder characterized by tremor, rigidity, bradykinesia, and a progressive loss of dopaminergic neurons from the substantia nigra containing Lewy bodies and Lewy neuritis surrounded by a halo constructed by α-syn (Forno et al., 1996). Filamentous α-syn has been shown to be the major component of these deposits that have been seen in the identification of mutations in the early onset of familial PD (Spillantini et al., 1998; Polymeropoulos et al., 1997). A study by Kirik et al., suggested that Parkinson-like degeneration is induced by a targeted over expression of α-syn using viral vectors in the nigrostriatal system in mice (2003).

Although the ability of α-syn to form fibrils has been demonstrated in numerous in vitro studies, the molecular mechanism of this process remains unclear. One possible mechanism in the formation of α-syn fibril involves a c-terminally truncated α-syn (Crowther et al., 1998). These truncated forms of α-syn (1-131, 1-120, 1-81) are able to assemble into filaments similar to those found in neurodegenerative diseases (Crowther et al., 1998; Uversky et al., 2001). Another mechanism points to the absence of significant secondary structure and the lack of hydrophobic core of α-syn, as viewed by UV spectroscopy (Kim, 1997; Weinreb et al., 1996), may lead to α-syn transformation from the normal soluble form of the monomeric protein into an insoluble conformation (Uversky et al., 1998). This is a critical step before fibril formation which is initiated by nucleation, followed by oligomerization, and leads to profibrils and finally fibrillization (Uversky et al., 1998). Recent studies have revealed that the accumulation of α-syn can result in immediate formation of different sizes and shapes of oligomers (Conway et al., 2000; Tsigenly et al., 2008;). These oligomers interact with lipids, disrupt membranes, and cause cell death in vitro in non-mammalian models (Conway et al., 2000). These α-
syn oligomers increase intracellular calcium levels, decrease cAMP response element-binding protein (CREB) transcriptional activity, and have a detrimental impact on neuronal welfare and memory functions in cultures neuroblastoma cells of the hippocampus (Martin et al., 2011). According to Martin et al., (2011) CREB inhibition and calcineurin induction were observed when α-syn oligomers were applied to brain slices. This interfered with hippocampal long-term potentiation and impaired memory through a calcineurin-dependent mechanism (2011). In fact, oligomer toxicity is exemplified in some forms of PD such as the juvenile form in the absence of Lewy body formation (Olanow et al., 2004).

A recent study has suggested that fragments of α-syn can associate with amyloid plaques and promote the aggregation of α-syn in the limbic regions of Alzheimer’s patients in vivo (Crews et al., 2009). Furthermore, it is speculated that α-syn oligimer accumulation specifically damages limbic structures that are associated with the neurodegenerative disorders such as Alzheimer’s disease and Parkinson’s disease and may cause memory deficits (Hashimoto et al. 2003; Braak and Braak 1999;). However a study by Lim et al., 2011, suggests the suppression of α-syn can cause the reversal of synaptic and memory defects in mouse models with dementia and lewy bodies (Lim et al., 2011). This may suggest that α-syn is likely to play a role in memory formation and plasticity (George et al. 2002; Lim et al., 2011) For this study, a novel object recognition (NOR) behavioral task, electrophysiology and methamphetamine as a pharmacological agent will be used to investigate the effects of α-syn on memory consolidation.
Alpha synuclein Fibrils

Figure 6: Electron micrograph of *in vitro* assembled α-syn fibrils. Taken from http://www2.mrc-lmb.cam.ac.uk/archive/papers/CP72-12.pdf
Specific Aims

The aim of this study was to determine if chronic METH administration causes molecular and behavioral modification associated with α-syn expression. Since α-syn accumulation has been implicated in memory impairment, we addressed the following:

(1) To assess the effect of chronic METH on memory consolidation and synaptic plasticity by using novel object recognition test (NOR) and Electrophysiology as assessment tools, and

(2) Evaluate if METH alters α-syn expression in the hippocampus

It is hypothesized that chronic METH interferes with memory formation partly as a consequence of drug-induced alterations in α-syn expression.
2. Materials and Methods

2.1 Animals

Twenty-nine male Hartley (HA) guinea pigs (2 weeks old; 200-250 g) were obtained from Charles Rivers Laboratory (Wilmington, MA). The animals were housed in groups of two per cage (18.5 x 18.5) at the Animal Care Facility (ACF) Center at Meharry Medical College (Nashville, TN). They were kept in a 12:12 hr light/dark cycle for at least 3 days prior to any drug treatment or experimental procedures. The ACF maintained the animals in temperature controlled rooms at 65-70 °F. The animals were housed for a maximum of 21 days total. The animals had access to food and water *ad libitum*. A maintenance record of each guinea pig was kept indicating date of arrival, weight, age, date used, and treatment regimen. Animals were processed in accordance with guidelines and regulations of the Institutional Animal and Care and Use Committee (IACUC) for the use of vertebrate animals.

2.2 Pharmacology

METH, obtained from Sigma (CAS # 51-57-0; St. Louis, MO), was administered to guinea pigs to determine its effect on recognition memory, synaptic plasticity and expression of α–syn in the hippocampus. Each guinea pig was weighed prior to surgery to calculate the appropriate amount of METH dosage to ensure that 10 mg/kg would be received throughout a 24 hour period through the azlet mini pump (Durect Corporation; Cupertino, CA). METH was dissolved in Phosphate Buffer Saline (PBS).
2.3 Surgery

Guinea pigs (n=29) were anesthetized with (4%) isofluorane as general anesthesia. Alzet mini-pumps (Durect Corporation; Cupertino, CA) were implanted subcutaneously on the back of the guinea pigs and were set to deliver a continuous infusion of METH (10 mg/kg; Sigma; St. Louis, MO) or saline (0.9% NaCl solution for 7 or 14 days. The incisions made in the skin were sutured closed with surgical staples and the guinea pigs were allowed to recover from the local anesthesia.

2.4 Treatments

The basic treatment designs in the studies are in Figures 7 – 9. All guinea pigs were used in Novel Object Recognition (NOR) (n=29) task after the appropriate treatment was given. Guinea pigs were randomly assigned to electrophysiology (n=6), molecular analysis (n=14), and the remaining animals were used for other studies. For the molecular analysis, guinea pigs were assigned to one of three experimental groups that were identified as (a) 7 day METH treatment, (i.e. 10 mg/kg/day; n=6), (b) Post Washout group, (i.e. 10 mg/kg; n=6), (c) Saline, (i.e. 0.9% NACL; n=2). The Post Washout group had 7 days of continuous METH infusion followed by 7 days of no drug (i.e. METH) treatment. The treatment groups (n=12) were subjected to treatment of high dose METH (10 mg/kg) per day as illustrated in Figure 7 while the saline group was subjected to treatment of saline (i.e. 0.9% NaCl solution; n=2). The appropriate treatments were administered using an Alzet osmotic mini-perfusion pump for a period of 7 or 14 days at a rate of 1.0 µl per hour or 24 µl of saline in each 24-hour period.
Figure 7. Treatment plan for all guinea pigs (n=29). All of the guinea pigs did go through NOR (n=29). For electrophysiology (n=6) and molecular analysis (n=14) animals were randomly assigned. Out of the METH set two animals went through electrophysiology and six were used for molecular studies. Out of the saline set two animals went through the electrophysiology and the remaining two were further assessed for molecular studies. Out of the Post METH group two animals went through electrophysiology and six were used for molecular studies.
7 day METH Experimental Design

Figure 8: Animals arrived on day 0 and were acclimatized for a period of 3-4 days. Alzet mini-pump implants were inserted on day 3. Drug treatments were performed on the same day for all guinea pigs (n=29). Guinea pig handling occurred on day 7-9 for the 7 day METH (n=10) and saline (n=2). NOR testing occurred on day 10. Euthanization of animals and tissue harvest occurred on days 11 and 12 for future molecular testing.

Post Washout Experimental design

Figure 9. Post washout period (n=12) occurred between days 9-15. GP handling for Post Washout (n=12) and Saline (n=3) occurred on days 16-18. NOR behavioral assessment occurred on day 19. Euthanization of animals and tissue harvest occurred on days 19-20 for further molecular studies.
2.5 Behavioral Assessment

The Novel Object Recognition test (NOR) is a behavioral task technique that is used to assess memory recall. Memory recall is a way to obtain information from your memory without having a cue or a trigger to activate past information or episode while recognition memory requires a trigger or a cue for memory activation of past information or episode (Haist et al., 1992). Recognition memory refers to the ability to judge a previously encountered item as familiar. Recognition memory is widely viewed as consisting of two components: recollection and familiarity (Malmberg, 2008; Atkinson and Juola, 1974). Recollection involves remembering specific contextual details about a prior learned episode and familiarity involves simply knowing that an item was presented, without having any available additional information about the learned episode. Tasks that assess recognition memory (and object recognition memory) in particular, have become increasingly useful tools for basic and preclinical research investigating the neural basis of memory (Winters et al. 2008). NOR consist of three phases: the familiarization phase, the delay phase, and the testing phase (Clark and Martin, 2005).

2.5.1 Guinea Pig handling/Habituation

All guinea pigs (n=29) were handled for 5 minutes each day for 3 consecutive days to allow acclimatization to the testing environment and to the researcher. After the handling phase, the guinea pigs were allowed to explore and become familiar with the empty Nodulus Phenotyper, Home Cages (18.5 x 18.5), designated as the testing arena, (Leesburg, VA) for 5- additional minutes. In all experiments, the Phenotyper home cages were cleaned prior to each phase of the experiment. See Figure 10 for diagram of Phenotyper.
2.5.2 Re-habituation

At the beginning of the trial period, each guinea pig was placed in a Nodulus Phenotyper Home Cage and was allowed to explored the cage for 5 minutes. Guinea pigs were removed and placed in a transport box for 15 minutes, and two identical objects were placed in the Nodulus Phenotyper Home Cage. See Figures 11 and 12 for experimental diagram for the NOR test.

2.5.3 Familiarization

The guinea pig was returned to the Nodulus Phenotyper Home Cage and allowed to explore the two identical objects for 5 minutes.

2.5.4 Delay

The guinea pig was removed from the Phenotyper Home Cage and taken back to his individual home cage for a 3 hour interval. During the delay phase a novel object along with one familiar object was placed in the Phenotyper Home Cage.

2.5.5 Testing

After completion of the 3 hour delay interval, the guinea pig was returned to the Phenotyper Home Cage and was given the opportunity to explore the two objects, one which was novel for 5 minutes. See Figures 11 and 12 for NOR experimental schematic.
Figure 10. Phenotyper where NOR assessment took place. The cage is 18.5” x 18.5” and equipped with a video tracker (A) which monitors the animal’s behavior and movement throughout the cage. In this diagram B indicates the water bottle for the animal, and C indicates the door in which the animals enter the phenotyper. Adapted from http://www.noldus.com/.
2.5.6 Processing

After the NOR tests were completed most of the guinea pigs were euthanized with an overdose of pentobarbital (150 mg/kg body weight i.p.). Both hippocampi were harvested and preserved in RNAse Later (Ambion; Camarillo, CA) for subsequent molecular studies. Of the remaining guinea pigs, six were randomly selected to be processed electrophysiologically to access synaptic changes in the hippocampus (see Electrophysiology section below).
Basic Novel Object Recognition Test

Figure 11. The basic experimental layout for the NOR behavioral assessment test for the guinea pig (n=29) includes the familiarization phase, the exploration of two identical objects for 5 minutes, and the test phase which includes the exploration of two objects, one of which is novel, for 5 minutes after a 3 hour delay.

Figure 12. Basic experimental layout for the NOR behavioral assessment test for the guinea pigs (n=29) includes habituation, familiarization and, the Nor test (administered after a 3 hour interval).
2.5.7 Analysis:

EthoVision XT (Nodulus, Virgina) software was used for the automatic tracking and analysis of animal movement, activity, and behavior in the Nodulus Phenotyper. To assess recognition memory, the amount of time spent exploring the novel object verses the familial object was evaluated. A discrimination index (DI) was calculated by subtracting the total time spent with the new object from the familiar object and dividing this value by the sum of the time spent exploring both objects. DI = (Time exploring NOV – Time exploring FAM)/ (Total time exploring NOV + FAM). A Positive DI values indicated a preference for the novel object which is suggestive of memory consolidation.
2.6 Electrophysiology

Guinea pigs were anesthetized with urethane (1500 mg/kg i.p.) dissolved in phosphate buffered saline (PBS) and were given Buprenorphine analgesic (.03 mg/kg; i.m.). To ensure that the animal was anesthetized, noxious stimulus such as pinching of the ear and hind limbs was done. If the animal twitched, withdrew a knee, or increased breathing was noticed, the animal was given a booster dosage of anesthesia, which was approximately 10-15% of the start dose. Each animal was firmly secured in a stereotaxic apparatus, with ear bars and mouth piece to stabilize the head. The top of the animal’s head was shaved and disinfected with alcohol wipes, after which, a subcutaneous injection of 2% lidocaine was injected under the skull as local anesthetic (0.2-0.3 mL). A circular patch of skin 2-3 cm was made to expose the skull. As needed hydrogen peroxide (30%) was used to clean the exposed skull to prevent further bleeding. The bregma was identified as the zero reference point for determining hippocampus coordinates for electrode placement. Small access holes (~2 mm diameter) were made in the skull to allow for the lowering of bipolar tungsten stimulating electrodes (SNEX-15, Rhodes) in the left CA3 region (-4.5 mm posterior, 5 mm lateral and -5.0 mm vertical relative to bregma) and in the left CA1 region (-5.0 mm posterior, 2.5 mm lateral and -3.5 mm vertical rise relative to bregma). Stimulation was applied to activate the CA3 neuron region and elicit population spike responses within the CA1 region. Once all electrodes were in place, recordings proceeded while monitoring vital signs. See Figure 13 for schematic of recording. Appropriate measures were undertaken to make sure each animal remained fully anesthetized. To maximize animal comfort, during the recording guinea pigs were blanketed with warming pads in order to maintain regular body
temperature. Animal experiments were performed with strict adherence to protocols approved by the Institutional Animal Care and Use Committee. Stimuli were adjusted (100 -150 µA for 0.01 msec at 1 Hz) to evoke CA1 field potentials with amplitudes between 0.5 -1.0 mV. This response was monitored for 30 minutes. After the 30 minutes of stable recording, a high frequency tetanic stimulation was applied to elicit LTP (100 Hz, 1sec, 3 trains at 60 sec intervals). The evoked population spikes were monitored for at least another 180 minutes at the original stimulation (100 -150 µA for 0.01 msec at 1 Hz). The recorded population spike and spontaneous field potentials were amplified at a gain of 1000 X and filtered between 1Hz and 5 kHz using an AC amplifier. All population spike recordings that were acquired during the sessions were stored in the computer using pCLAMP10 software. At the end of the recordings, the guinea pigs were euthanized with an overdose of pentobarbital (150 ml/kg body weight i.p.). The whole brain was immediately removed and stored in isopentane for subsequent molecular studies (i.e. in situ or Western blot).
Electrophysiology Recording

Figure 13. An *in vivo* animal model. Stimulation in the left CA3 area evokes population spikes in the left CA1 sub-fields. We recorded spontaneous and evoked population spike in the left CA1 area only.
2.7 Analysis

The basic parameters were extracted from the recorded field potentials and this included amplitude, onset latency, and duration. However only the change in the amplitude as a function of time was analyzed. The field potential typically has a positive-going wave (PW1) that is interrupted by a negative-going population spike (PS), followed by another positive-going wave (PW2). PW1 reflects synaptic depolarization whereas PW2 corresponds to the input to CA1 cells (Anderson et al., 1964). The PS is due to a summation of discharging of CA1 neurons (Anderson et al., 1971). The basic criterion for the detection of late-LTP was enhancement in post-conditioning synaptic responses, lasting beyond 2 hrs (one-way ANOVA with Scheffe’s test as the a posteriori test ($\alpha < 0.05$)).

2.8 Molecular Studies:

2.8.1 Protein Isolation

The total protein was isolated from frozen hippocampal tissue samples previously stored in RNA later. The tissue sample is first washed in PBS and the weight of the tissue sample was recorded before homogenization. About 600 $\mu$L (or 6-8 volumes per tissue mass) of ice-cold Cell Disruption Buffer (Invitrogen; Camarillo, CA) was added into a tube with the tissue sample and homogenized using the Pro 200 motor rotor-stator (Invitrogen; Camarillo, CA). The mixture was centrifuged at 16,000 x g for 20 minutes at 4° C. The pellet was then discarded and the supernatant, was aliquoted and stored until used.
2.8.2 Protein Concentration Determination via Bicinchoninic Acid Assay

Protein concentration was measured using the Pierce Bicinchoninic Acid (BCA) Protein Assay as described by the manufacturer (Thermo Scientific, Rockland, IL). Bovine serum albumin (BSA) was used as a standard. This technique is based on the reduction of Cu$^{++}$ to Cu$^{+}$ by protein in an alkaline medium as follows:

1. Protein (peptide bonds) + Cu$^{++} \rightarrow$ tetradentate-Cu$^{+}$ complex
2. Cu$^{++} + 2$ BCA $\rightarrow$ BCA-Cu$^{+}$ complex (purple color)

This purple color reaction product exhibits a strong absorbance at 562 nm that increases linearly with increasing protein concentration over a broad range of 20-2000 µg/ml. To determine the sample concentration, a plot was made of the values of BSA verses their known concentrations (ranging from 0 to 2000 µg/ml). A linear regression line was fitted from which the slope coefficient was calculated. At the same time several dilutions of each sample were also assayed and the calculation was obtained by taking the mean scored and dividing by the slope coefficient obtained from the BSA standard plot to obtain protein concentration for each sample.

2.8.3 Protein Analysis

Protein bands were separated according to their weights using the standard procedures of the NuPage MES kit on a precast 4-12% NuPAGE Novex Bis-Tris Gel (Invitrogen; Camarillo, CA). Individual samples were made according to the constituents shown in Table 1. Samples were heated for 10 minutes at 70°. Proteins were electrophoresed and in duplicates using the MES Running Buffer as shown in Table 3.
SDS-PAGE separation occurred for 35 min at 200V. After the completion of electrophoresis, the gels were transferred on a polyvinylidene fluoride (PVDF) membrane at 25V for 60 minutes using the Novex XCell blotting apparatus. The blotting sandwich was prepared and carefully inserted into the 1X transfer module in the Novex XCell unit with the transfer buffer. See Table 2 and Figure 14.
**NUPAGE Reduced sample preparations**

Table 1. Preparation of Reduced samples for denaturing gel electrophoresis using the NuPAGE Novex Bis-Tris

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>20 μL</td>
</tr>
<tr>
<td>NuPAGE® LDS Sample Buffer (4X)</td>
<td>2.5 μL</td>
</tr>
<tr>
<td>NuPAGE® Antioxidant Reducing Agent</td>
<td>1 μL</td>
</tr>
<tr>
<td>Deionized Water to</td>
<td>6.5 μL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>10 μL</td>
</tr>
</tbody>
</table>

**NUPAGE MES Running buffer**

Table 3. Preparation of the Running buffer.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NuPAGE SDS Running buffer (20X MOPS)</td>
<td>50 mL</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>950 mL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1,000 mL</td>
</tr>
</tbody>
</table>

**NUPAGE MES Reduced Transfer buffer**

Table 2. This transfer buffer was used during the transfer phase to ensure maximum protein transfer from the gel to the PVDF membrane.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>NuPAGE Transfer Buffer (20X)</td>
<td>50 mL</td>
</tr>
<tr>
<td>NuPAGE® Antioxidant</td>
<td>1 mL</td>
</tr>
<tr>
<td>METHanol</td>
<td>100 mL</td>
</tr>
<tr>
<td>Deionized Water</td>
<td>849 mL</td>
</tr>
<tr>
<td>Total Volume</td>
<td>1,000 mL</td>
</tr>
</tbody>
</table>
Figure 14. Depiction of Novex XCell II blotting apparatus set-up. Transfer sandwich was built from the base up with two blotting pad, filter paper, gel, PVDF transfer membrane, filter paper, and lastly blotting pad.
2.8.4 Western Blot Procedure for Alpha Synuclein

After the removal of the PVDF membrane from the transfer buffer, the membrane was washed briefly with 1X Tris-Buffered Saline and 10% Tween-20 (TBST). The membrane was rinsed off and incubated in blocking solution (10 ml of 5% milk in TBST) for 1 hour. 15 µl of polyclonal rabbit α synuclein (; Santa Cruz Biotechnology; Santa Cruz, CA, dilution 1:200) was added to 10 ml of 5% milk and the membrane was incubated overnight at room temperature. The following morning, the membrane was washed in three washes of TBST, 5 minutes each, and incubated with anti-rabbit secondary antibody conjugated to horseradish peroxidase (Bio-Rad, Hercules, CA) in 5% milk in TBST blocking solution for 1 hr at room temperature. After the incubation, the membrane was washed in three washes of TBST, 5 minutes each. For signal development, the Detection Reagent Working Solution, Reagent 1 and Reagent 2, were used from the Fast ECL kit (Thermoscientific, Rockland, IL). After mixing the Detection Reagent Working Solution in a 1:1 dilution, the solution was poured onto the membrane (approximately 3 ml of mixture per membrane). The detection reagent working solution interacted with the membrane for about 1 minute. The membrane was removed from the detection solution and was wrapped in a clear plastic wrap. The membrane was placed in a cassette tape and developed on film in a dark room for the appropriate time of 1 minute. Figure 15 shows a schematic of the Western blot procedure.

After film exposure, the membrane was washed with TBST for 5 minutes to rinse off the detection agent. Membrane was re probed with 0.5 µl GAPDH mouse monoclonal (Life Technologies; Camarillo, CA) in 10 ml of 5% milk/TBST incubated overnight at room temperature. Then, the membrane was removed from the antibody
solution and washed three times in TBST for 5 minutes. The membrane was then incubated with 0.5 µl anti-mouse monoclonal (Santa Cruz Biotechnology; Santa Cruz, CA) for loading control purposes in 10 ml of 5% milk/TBST and immunoreactivity was visualized with the Fast ECL kit (Thermoscientific, Rockland, IL) as described above. After development and exposure, the film was scanned and analyzed using the AlphaEase FC 4.0 (Alpha Innotech; San Leandro, CA).
Western Blot Procedure for Alpha Synuclein

1) Protein Isolation/ Determine protein concentration (BCA Kit)

2) Electrophoresis
   Settings: 35min, 200V, 125 mA

3) Transfer on PVDF membranes
   Settings: 30V, 60 minutes, 125 mA

4) Block for an 1 hour 10 ml of 5% milk/TBST

5) Added polyclonal rabbit primary antibody (15µl per 10ml of 5% milk/TBST) overnight

6) Washes/ anti-rabbit secondary antibody conjugated HRP (5µL per 10ml of 5% milk) 1 hr

7) Wash/Expose to Detection Reagents from Fast ECL kit/ expose to film and analyze data

8) Strip/Add .5µl GAPDH mouse monoclonal primary antibody to 10ml of 5% milk/TBST

9) Wash/Add .5µl on anti-mouse monoclonal as secondary in 10ml of 5% milk/TBST

9) Wash/Expose to Detection Reagents from Fast ECL kit/ expose film and analyze with loading control

Figure 15. Flow Chart depicting molecular procedures used to assess α-syn expression in the guinea pig hippocampus after drug treatment with METH.
3. Results

3.1 NOR Behavioral Assessment

Twenty-nine animals were used to access recognition memory in the hippocampus. The technique used in this evaluation was the Novel Object Recognition (NOR) task that evaluated recognition memory, the amount of time spent exploring the novel object versus the familial object. NOR was evaluated by using the Discrimination Index (DI). A discrimination index (DI) was calculated by subtracting the total time spent with the new object from the familiar object and dividing this value by the sum of the time spent exploring both objects. Figure 16 shows the discrimination index (DI) for the novel object for each subgroup (i.e., Saline, Post Washout, and METH). Exploration of the objects by the guinea pigs (n=29) included sniffing, touching or time when the head region was within a defined perimeter around the object (FAM or NOV etc). On testing day the interval between familiarization and NOR testing was 3 hours. Both the Saline group and the Post Washout group exhibited a preference for the novel object on the NOR task as indicated by a positive DI. The important finding was that the Post Washout group exhibited preference for the novel object, but the METH group only showed a preference for the familiar object. The overall pattern of exploration through the NOR test was different in the three groups, but the Saline group (0.63 +/- 0.05) exhibited a much stronger preference for the novel object than did the Post Washout (0.15 +/- 0.06). Importantly, the METH group did have a preference for the familiar object (-0.21 +/- 0.07) as shown in Figure 16. In fact, the METH group had a negative discrimination index which suggests this group did not have a preference for the novel object. This
affect by the METH group was statistically significant when compared to the saline groups (p > 0.05) using the 1-way ANOVA with Tukeys post-hoc tests.
Figure 16. A positive DI indicates a preference for the novel object and our results show guinea pigs in the saline group and Post Washout obtained positive scores on the NOR, i.e. showed preference for the novel object. While the METH group obtained a negative score on the NOR, i.e. did not show a preference for the novel object.
3.2 Electrophysiology

3.2.1 Evoked Potentials

Guinea Pigs (n=6) were used to evaluate the synaptic plasticity across the CA3/CA1 region of the hippocampus. CA3 afferent were stimulated to evoke population spikes in the CA1 region. For data analysis, the criteria for animal inclusion included recordings that exhibited stable evoked population spikes monitored for at least 30 minutes. The evoked field potentials were typically comprised of an initial positive-going wave (PW) that was interrupted by a negative-going population spike (PS) followed by a second PW (Fig. 2). The first PW primarily reflects synaptic depolarization whereas the second PW corresponds to inhibitory influences occurring in CA1 cells. The population spike is due to the summation of CA1 neurons discharging, and we quantified it as ‘the vertical rise from peak negativity to the intersection point with a line drawn at a tangent between the two PWs’. Samples of evoked potentials (n=3) are shown in Figure 17. In all treatment groups pre-tetanus responses were successfully induced. With LTP taken to be at 40% increase in post tetanus potentials over baseline responses. It can be deduced that LTP was successfully induced in all groups at 60 minutes. The maintenance (180 minutes) of the response was however not pronounced in the METH treated animals. The population spike amplitudes were not similar among the different animal groups and exhibited amplitudes ranging between 3-8 mV for 7-day METH treated animals, 3-12 mV for the Post Washout animals, and 9-24 mV for Saline animals.. Taken together the high frequency tetanic stimulation (100 Hz, 1 sec, three trains at 60 sec intervals) produced both early-LTP and late-LTP in the Saline, Post Washout and METH groups.
LTP across the CA3/CA1 region was defined as 40% greater than pre-tetanus levels (100%). Results indicated that LTP was induced in all animal groups (n=3) but was more robust in the Saline treated animals (Figures 18-21). LTP was classified as (1) early-LTP (1-2 hours) and (2) late-LTP lasting beyond 2 hours. Presented in Figures 18-20 are individual time plots of population spikes for Saline (n=1), 7 day METH (n=1) and Post Washout (n=1). Figure 21 depicts the combine time plot of all treatment groups (n=3).
Figure 17. Evoked population spikes showing LTP in the different guinea pigs (n=3) hippocampus in vivo. The basic recording comprised of an initial positive-going wave (PW) followed by second PW. Population spike amplitude was measured as the vertical rise from peak negativity to the intersection point with a line drawn at a tangent between the two PWs.
Figure 18. Saline time plot of LTP across CA3/CA1 synapses in guinea pig hippocampus *in vivo*. The graph is a plot of individual population spikes over the duration of the experiment. Usually the pre tetanus response (last ten) is averaged together to get a pre-tetanus baseline response. Briefly, LTP was observed as significant population spike enhancements of at least 40% above pre-tetanus baseline responses. Early-LTP and late-LTP was developed and maintained in the saline group (n=1).
Figure 19. Time plot of the 7 day METH. LTP across CA3/CA1 synapses in guinea pig hippocampus in vivo. The graph is a plot of individual population spikes over the duration of the experiment. Usually the pre tetanus response (last ten) is averaged together to get a pre-tetanus baseline response. Briefly, LTP was observed as significant population spike enhancements of at least 40% above pre-tetanus baseline responses. Early-LTP and late-LTP was developed and maintained in the METH group (n=1).
Figure 20. Time plot Post Washout of LTP across CA3/CA1 synapses in guinea pig hippocampus *in vivo*. The graph is a plot of individual population spikes over the duration of the experiment. Usually the pre-tetanus response (last ten) is averaged together to get a pre-tetanus baseline response. Briefly, LTP was observed as significant population spike enhancements of at least 40% above pre-tetanus baseline responses. Early-LTP and late-LTP was developed and maintained in the Post METH group (n=1). L-LTP was more robust in Post Washout treatment group than the 7 day METH group.
Figure 21. A combined time plot of LTP across the CA3/CA1 synapses in the guinea pig (n=3) hippocampus *in vivo*. Briefly, LTP was observed as significant population spike enhancements of at least 40% above pre-tetanus baseline responses. Early-LTP and late-LTP were developed and maintained in all animal groups (n=3). However, L-LTP was more robust on the Saline-only control group.
3.2.2 Western blot

For the quantitative assay, equal amounts of protein from the 3 treatment groups were electrophoresed concurrently. Samples were from taken from the 7 day METH (n=6), Post Washout (n=6), and Saline (n=2) groups. The samples were transferred onto the PVDF membranes, blotted with α-syn, developed and exposed to film as described in the Method section. In Figure 22 we found that hippocampal protein samples probed with polyclonal rabbit α-syn primary antibody (Santa Cruz Biotechnology; Santa Cruz, CA) yielded specific bands that matched the expected size of guinea pig α-syn at 17kda. We determined the densitometric measurements from the Western blots by using the Alpha Ease FC 4.0 software, normalized against GAPDH and charted the results using the Alpha Ease FC 4.0 software. Figure 22 showed the α-syn band expression of a single gel. Figure 23 shows the histogram of the average densitometric measurement of individual α-syn bands. The results (Figures 22 and 23) indicate higher levels of α-syn present in the hippocampus of Saline-treated animals (n=2), and slightly decreased levels in the Post Washout (n=6) and the METH groups (n=6). Taken together, our data raised the prospect that there was a down regulation of endogenous α-syn protein levels in the hippocampus between subgroup classifications; however, these differences were not statistically significant.
Figure 22. Quantification of endogenous α-syn protein levels relative to the loading control (GAPDH). Each band within a subgroup represents an individual guinea pig.
Alpha Synuclein Densitometry

Figure 23. Western blot depicts α-syn expression normalized to the control GAPDH, taken from the 7 day METH (n=6), Post Washout (i.e. 7 Day METH with WO; n=6), and Saline (n=2) groups. The Saline group (n=2) shows a higher densitometry.
4. Discussion

In the present study we investigated the memory performance of guinea pigs (n=29) under varying METH treatments. The METH group was continually exposed to METH (10mg/kg) per day for 7 days (n=10), Post Washout (n=12) was continually exposed to METH (10 mg/kg) per day for 7 days followed by 7 “washout” days, and the Saline group (n=7) was administered saline for 14 days and served as the control for this experiment. We assessed the behavioral component through a NOR test and the plasticity component through an Electrophysiology test to determine if memory consolidation occurred in the animals. Additionally, we determined the levels of α-syn, a biochemical marker that is implicated in neurodegenerative diseases such as Alzheimer’s and Dementia Lewy bodies in the hippocampus, a brain region known to be involved in the memory function. The key trends are that continual exposure to high dose METH results in a loss of object recognition.

During the NOR behavioral assessment test, the guinea pigs (n=29) were given 5-minute exposures to objects during the habituation, familiarization and the NOR phase and a discrimination index (DI) was assessed. We determined time spent exploring each object for the first 3 minutes during the different phases (i.e. Familiarization and NOR) as applicable to calculate the DI. Finally we calculated the DI particularly between novel object (NOV) and familiar object (FAM) as follows: DI = (Time exploring NOV – Time exploring FAM)/ (Total time exploring NOV + FAM). Positive DI values indicated a preference for the novel object which is suggestive of memory consolidation. After the NOR test was assessed, the Saline group (n=7; DI= 0.63+/−0.05) had the strongest preference for the novel object followed by the Post Washout group (n=12; DI= 0.15 +/-
0.06) and finally the METH group (n=10; DI= -0.21 +/- 0.07). As a consequence, we were able to obtain a reliable and robust measure of performance for the Saline group (n=7). For future studies, an increase in the delay time from 3 hours to 24 hours or longer can be used to see if memory consolidation was maintained between guinea pig subgroups. It is worth noting that in studies by Luparini et al., (2000) when the period between the presentation of objects was increased (24 h) these groups lost the ability to recognize different objects. Increasing the time between the presentation of the familiar and the new object rendered animals unable to recognize novelty beyond 3 hours.

In determining the effect METH has on synaptic plasticity, a key finding was in the maintenance of late-LTP in animals treated with chronic METH (n=2). L-LTP was achieved and was not maintained can be attributed to (1) too few animals were used (n=2) and (2) the continuous infusion of METH in the brain may lead to a saturation effect. However, when there are not enough animals used for the study only trends can be suggested. One possible trend from the data is that METH treated animals may not maintain late-LTP. Additionally, due to the continuous infusion of METH, a saturation effect may have affected the process that lead to late-LTP. One possibility is that METH has desensitized the dopamine receptors. It is interesting to note that rats that have been treated chronically with METH for ninety days could not produce LTP (Chirwa et al., 2005). It is suggestive from this data that the early and late phase of LTP was induced and but only the late phase was not sustained. The second key finding is the POST Washout group (n=2) that went through 7 days of continuous METH treatment followed by 7 days of “washout” which also showed the L-LTP was not maintained. The early phase of LTP was also induced in the hippocampus, but the late-LTP was not sustained
What is interesting in this group is that they showed a positive DI for the novel object during the NOR test which could suggest that after a “washout” period of METH memory recognition can be achieved. However, the guinea pig did show some signs of late-LTP but could not sustain it. A very likely possibility is small sample size of the group (n=2) did not allow for sufficient data to be collected. A large group will give more information that will lead to a more definitive conclusion.

In terms of molecular analysis of the guinea pig hippocampus expression, the focus was α-syn for several reasons including the implication of this protein in neurodegenerative diseases such as Alzheimer’s and Parkinson’s disease whose major symptom is memory loss. According to Kokhan et al., at the molecular and cellular level α-syn deficient models may result in impairment of memory formation with aging animals (2011). Another study reported that abnormal accumulation of α-syn in the limbic system, particularly in the hippocampus, correlated with memory impairment and led to structural synaptic deficits (Lim et al., 2011). Furthermore, when α-syn expression was suppressed, partial clearing of pre-existing α-syn pathology and reversal of structural synaptic defects was observed, resulting in an improvement in memory function (Lim et al., 2011). Interestingly when α-syn expression was investigated in the hippocampus of the guinea pigs for METH (n=6), Post Washout (n=6) and Saline (n=2) there was no statistical significance in the results once it was normalized with the loading control GAPDH. For instance, the present study did not measure the level of α-syn expressed in the guinea pigs before the experimental procedures. Additionally, the lack of significance seen in α-syn could be due to reduce sensitivity of the protocol. Also more sensitive tools are needed such as probes with oligomers that are more specific and
sensitive to α-syn in order to make a definite conclusion or comparison. Another reason for the nature of protein expression could be the fact that METH can possibly alter α-syn through post-translational modification on different parts of the protein because α-syn can be over or under expressed. A recent study by Lim et al., (2011) demonstrates the suppression of α-syn expression can lead to the memory improvement. It is also a possibility that METH does not have an effect on α-syn expression since the function of α-syn is not widely known. We should have assessed protein or gene expression of α-syn through different points in the experiment to establish a pattern of change instead of the one point that we focused on. A-syn that is usually seen in neurodegenerative diseases in over expression or mutation could be trigged by other properties or mechanism in the brain that causes the abnormality seen in neurodegenerative disease. With regards to this project, the sample size was too small in order to effectively evaluate a mechanistic route for METH, α-syn, and memory loss connections. This is only a stepping stone for the effects of chronic METH on biochemical markers such as α-syn.

In summary, the present findings suggests chronic METH administration has an affect on some of the same mechanisms underlining memory function and the drug-induced activation lead to some form of memory impairments and the role of alpha synuclein in the process of memory consolidation still remains unclear.
References


Behavioral and neurochemical effects of a 72-h binge. *Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology, 34*(11), 2430-2441.


dopamine uptake sites following repeated administration of methamphetamine. 
*Brain Research, 181*(1), 151-160.


