Genetic and Behavioral Discrimination of Dopamine 1 and 5 Receptors in Hippocampal Dependent Memory Consolidation

by

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Abstract

Dopamine (DA) containing neurons project throughout the brain. DA has been implicated in mediating brain disorders such as Schizophrenia, Parkinson's disease, Huntington's disease and drug addiction. The role of DA in working memory and procedural learning is also well established. DA is a ubiquitous neurotransmitter that affects much of the brain, but very little is known how dopamine functions in hippocampal dependent learning. It was only until recently that dopamine-containing neurons were found to project to the hippocampus. Even less understood are the expression patterns of DA receptors within the hippocampus and this is underlined by the inability of distinguishing the dopamine 1 receptor family (D1 and 5 receptors (D1/D5Rs)). Given the interaction of the D1 family with similar G-protein coupled receptors it has been assumed that these two receptors function in an analogous fashion. Additionally, the specific expressional pattern of each receptor lacks clarity due to non-specific binding by molecular probes. Moreover, D1 and D5 pharmacological and global KO studies cannot and have not functionally delineated D1Rs from D5Rs and global KOs of the D1Rs or D5Rs are not specific to the hippocampus, thus compensatory mechanisms likely ameliorate most physiological and behavioral deficits. Still, the aforementioned studies do point to the D1 family in modulating hippocampal synaptic plasticity, learning and memory consolidation. In order to characterize D1Rs distinctly from D5Rs we have generated three strains of conditional mutant mice (D1 KO, D5 KO, D1/5 KO). I present data that shows distinct expression patterns within the hippocampus, the importance of D1Rs and D5Rs in modulating hippocampal plasticity, and hippocampal dependent learning. These data highlight distinct functional roles of D1Rs and D5Rs in hippocampal function.

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Introduction: Multi-Level Analysis of Hippocampal Dependent Learning and Memory

Introduction: The Hippocampus and Learning

The hippocampus is a crucial brain structure in the formation and storage of declarative episodic memories (Scoville, Milner et al. 1957). The surgical removal of the medial temporal lobe (MTL) in the patient Henry Gustav Molaison (HM) showed that removal of the MTL, which includes the hippocampal formation, results in anterograde amnesia and prevents the formation of new episodic memories (Scoville and Milner 1957). Early research on HM provided evidence that lesions of the hippocampus affect memories of the recent past to a greater extent than memories of the remote past, highlighting the importance of the hippocampus as the initial sight of episodic memory formation and retention (Scoville and Milner 1957). Patient HM's surgery was formative to the field of learning and memory and pointed this field to the medial temporal lobe, however the precise locus of learning and memory was still unknown. It took the use of induced lesion studies in primates and rodents as well as further human research with patients that had focal hippocampal lesions, before the hippocampus was placed as the center of episodic and spatial memory formation. (Morris, Garrud et al. 1982; Zola-Morgan, Squire et al. 1986; Zola-Morgan, Squire et al. 1992). By having a precise area to study, the use of animal models in lesion studies started to grow tremendously, particularly in rodents where the use of pharmacological and genetic lesions allowed for the study of the molecular, cellular and behavioral

mechanisms that underlie hippocampal memory formation (Morris, Garrud et al. 1982; Morris, Anderson et al. 1986; Tsien, Huerta et al. 1996).

Memory Acquisition, Consolidation and Retrieval

The original finding with patient HM, that memories of the recent past are most affected by MTL lesion as compared to remote memories, opened up the study of memory consolidation, the process by which memories stabilize. The study of memory consolidation further delineated the process of memory formation into memory acquisition, consolidation and retrieval. Characterization of memory acquisition, consolidation and retrieval is studied by utilizing lesions techniques of the hippocampus during hippocampal forms of learning, such as classical conditioning. Ivan Pavlov's experiments on the digestive system of dogs unexpectedly led to the phenomenon of the conditioned reflex, termed classical conditioning (Pavlov 1927; Duncan 1949). Classical conditioning is a type of associative learning where a neutral stimulus, called the conditioned stimulus (CS), is associated with a stimulus of value, called the unconditioned stimulus (US). The US results in an unconditioned response (UR) and after CS-US pairings the CS alone can result in the conditioned response (CR). Classical conditioning has been utilized in understanding the mechanisms of hippocampal learning and memory using contextual fear conditioning, where an animal (such as a rodent) associates a distinct context (CS) with an aversive stimulus, often in

the form of a foot shock (US) (Scoville, Milner et al. 1957; Blanchard and Blanchard 1969; Kim and Fanselow 1992; Phillips and LeDoux 1992). During training the animal is placed in a context and is presented with a foot shock, the animal expresses the UR to the shock in the form of bursts of motor activity followed by bouts of freezing (CR). After training when the animal is placed back into the context the animal will freeze, and freezing is used as an index of memory (Blanchard and Blanchard 1969; Fanselow 1980). During the learning event, memory acquisition and initial consolidation is in a labile state where it is easily disrupted (G.E. and Pilzecker 1900; Duncan 1949; Agranoff, Davis et al. 1966). Learning events form in short term memory (STM) where the memory lasts on the time scale of seconds to hours and memories that consolidate become stable and enter into long-term memory (LTM) and last on the time scale of hours to a lifetime (McGaugh 2000). Lesion of the hippocampus prior to contextual fear conditioning hinders the CS-US association resulting in freezing deficits to the context, providing evidence the hippocampus is important in acquisition (Kim, Rison et al. 1993; Wiltgen, Sanders et al. 2006). Similarly, hippocampal lesions made after the training event abolishes contextual fear memory, highlighting the importance of the hippocampus in memory consolidation and retrieval (Kim and Fanselow 1992). However, lesions made to the hippocampus months after training do not impair memory providing evidence that memories are mediated by area outside of the hippocampus (Kim and Fanselow 1992). These results show that the hippocampus is engaged in the

initial acquisition of memories and overtime these memories of the recent past consolidate and eventually become independent of the hippocampus integrating into cortical regions (Zola-Morgan and Squire 1990; Kim and Fanselow 1992; Frankland, Bontempi et al. 2004). Greater understanding of memory formation came by way of physiological studies that generated new ideas that could function as the basis of learning and memory, primarily through synaptic plasticity, where the molecular components not only showed to be important in plasticity but also in memory formation.

Pharmacological Studies of Hippocampal Plasticity

Donald Hebb was the first to hypothesize that the change in communication between neurons lies as the basis for associative learning and is today known as Hebb's Postulate (Hebb 1949):

> When an axon of cell A is near enough to excite cell B and repeatedly or persistently takes part in firing it, some growth process or metabolic change takes place in one or both cells such that A's efficiency, as one of the cells firing B, is increased.

The initiation of metabolic changes that were hypothesized by Hebb were able to be directly tested when Bliss and Lømo discovered that when a strong current given to a hippocampal slice results in long lasting physiological changes, considered today to be the basis of associative learning and memory formation

(Bliss and Lomo 1973). The long lasting changes are in the form of increased efficacy of neuronal transmission at the synapse and is termed associative long-term potentiation (LTP). LTP was first established at the medial perforant path (mPP)-dentate gyrus (DG) synapse and it was later shown that the induction of LTP relied on activation of the N-methyl-D-aspartate receptor (NMDAR) (Collingridge, Kehl et al. 1983; Morris, Anderson et al. 1986). It was also found that NMDAR dependent LTP also occurs at the CA3-CA1 synapse (Collingridge, Kehl et al. 1983). The NMDAR functions as a coincidence detector, whereby a presynaptic input that co-occurs with a postsynaptic response is needed for removal of the Mg^{2+} blockade from the NMDAR pore allowing for the passage of Ca^{2+} into the postsynaptic neuron (Nowak, Bregestovski et al. 1984; Seeburg, Burnashev et al. 1995). Elevation in postsynaptic Ca^{2+} is necessary for the induction of LTP as Ca^{2+} activates protein kinase pathways that lead to the maintenance and expression of LTP (Lynch, Larson et al. 1983; Malinow, Madison et al. 1988; Malenka, Kauer et al. 1989). One such protein that has been shown to be critical in hippocampal plasticity is α-calcium-calmodulin protein kinase II (α-CaMKII), which is activated when Ca^{2+} enters into the postsynaptic neuron (Malenka, Kauer et al. 1989). Two forms of LTP have been reported; an early and late-phase LTP (E-LTP and L-LTP, respectively) where E-LTP relies on postsynaptic protein kinase activation, such as α-CaMKII, and L-LTP relies de novo protein synthesis (Krug, Lossner et al. 1984; Stanton and Sarvey 1984; Malenka, Kauer et al. 1989). The mechanism of L-LTP de novo

protein synthesis depends on the initiation of genetic transcription by the transcription factor cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). CREB activation occurs downstream from protein kinases activation, such as α-CaMKII, and when activated binds to specific DNA sequences called cAMP response elements (CREs), activating genetic transcription. The induced protein products are necessary for L-LTP induction (Sheng, Thompson et al. 1991; Bourtchuladze, Frenguelli et al. 1994). Thus, CREB is activated through Ca^{2+} influx by way of neuronal activity; CREB also acts as a convergent sight for cAMP inducted transcription, which can be initiated by activation of dopamine receptors (Forn, Krueger et al. 1974).

It has been well established that the neuromodulator dopamine can induce L-LTP in the hippocampus and that blockade of dopamine receptors blocks L-LTP in the hippocampus (Frey, Matthies et al. 1991; Huang and Kandel 1995; Navakkode, Sajikumar et al. 2007). The stimulation of dopamine 1 and 5 receptors (D1/D5Rs) activates adenylyl cyclase (AC) resulting in increased concentration of cAMP, cAMP activates protein kinase A (PKA), which initiates CREB mediated transcription (Kebabian and Greengard 1971; Kebabian, Petzold et al. 1972; Forn, Krueger et al. 1974; Creese, Sibley et al. 1983; Sheng, McFadden et al. 1990; Frey, Matthies et al. 1991). Moreover, D1/D5R activation increases Ca^{2+} currents through the NMDAR and indirectly increases Ca^{2+} through voltage gated calcium channels (Missale, Nash et al. 1998; Lee, Xue et al. 2002). What we can

take away from past research is that CREB induced transcription is pivotal in protein dependent synaptic plasticity and it is a convergent sight for Ca^{2+} and cAMP. Additionally, D1/D5R activation leads to both increased Ca^{2+} and cAMP concentration and is both necessary and sufficient for L-LTP emphasizing the importance of dopamine in synaptic plasticity. Pharmacological studies have provided a wealth of evidence by elucidating specific receptors, molecular and genetic pathways in synaptic plasticity (Collingridge, Kehl et al. 1983; Malenka, Kauer et al. 1989; Sheng, McFadden et al. 1990; Frey, Huang et al. 1993; Huang and Kandel 1995). The same pharmacological manipulations used in hippocampal physiology experiments have also increased the understanding of hippocampal dependent behaviors such as classical conditioning and spatial learning studies where many of these receptors and molecular events overlap (Morris, Garrud et al. 1982; Morris, Anderson et al. 1986; Silva, Stevens et al. 1992; Bourtchuladze, Frenguelli et al. 1994; Inoue, Izumi et al. 2000).

The Molecular Basis of Learning and Memory

NMDARs mediate synaptic plasticity and acquisition of hippocampal dependent learning and memory. Utilization of electrolytic and excitotoxic lesions disclosed the importance of hippocampal acquisition, consolidation and expression of conditional learning. However, gross lesions of brain systems overlook the complexity molecular events that further delineate specific aspects of memory

formation. With the use of pharmacological agents the receptors, molecular pathways and genetic networks in memory acquisition, consolidation and expression can be parsed out. Physiological studies of the hippocampus suggested that synaptic plasticity might play a role in memory formation (Bliss and Lomo 1973; Collingridge, Kehl et al. 1983). LTP studies at the mPP-DG and CA3-CA1 synapses shows that the NMDAR is necessary for the induction of LTP and based on these results the role of the NMDAR in acquisition of hippocampal dependent learning and memory, such as contextual fear conditioning, was tested by infusion of the NMDAR antagonist, (2R)-amino-5-phosphonopentanoic acid (APV), into the ventricles of rats (Morris, Garrud et al. 1982; Collingridge, Kehl et al. 1983; Morris, Anderson et al. 1986; Kim, DeCola et al. 1991). Ventricular infusion of APV, which blocks NMDAR in the hippocampus as well as other brain regions, resulted in impaired acquisition of the CS-US association as assessed by reduction of freezing when animals were tested. When APV is injected just prior to testing there are no deficits, providing evidence that hippocampal NMDARs are necessary for acquisition but not the expression of contextual fear (Kim, DeCola et al. 1991). In additional to contextual fear conditioning, the role of NMDARs was tested in hippocampal dependent spatial learning and memory through the use of the Morris water maze. Rats placed in opaque water search for a hidden escape platform in a large pool. Using distal cues placed around the maze, rats learn to associate the location of the hidden platform to the orientation of the distal cues. Morris showed that intra-ventricular

infusion of APV into rats prevented animals from correctly recalling the location of the escape platform during a probe test, however animals did show the ability to find the platform during training (Morris, Anderson et al. 1986). APV studies in classical conditioning and water maze show that the NMDAR is necessary for memory acquisition and subsequent memory consolidation, however NMDARs are not necessary for memory expression. However, ventricular injection of APV affects regions beyond the hippocampus and these studies cannot exclude the possibility that NMDAR blockade outside the hippocampus play a role in these behavioral deficits. Nonetheless, these results mimic the cellular correlates of learning and memory as exhibited in E-LTP studies where NMDARs are necessary for LTP induction but not maintenance or expression (Collingridge, Kehl et al. 1983).

D1/D5Rs mediate protein synthesis dependent consolidation in the hippocampus. It was noted above that NMDARs pass Ca^{2+} ions through its pore and can initiate molecular cascades that lead to transcription though CREB activation. CREB also serves as a convergent site for Ca^{2+} and cAMP. Activation of D1/D5Rs increases both Ca^{2+} and cAMP and is both necessary and sufficient for L-LTP (Huang and Kandel 1995; Missale, Nash et al. 1998). L-LTP relies on CREB mediated transcription for de novo protein synthesis and is thought to represent the cellular correlate of long-term memory, which also requires protein synthesis and shown to be CREB dependent (Flexner, Flexner et al. 1963; Agranoff, Davis

et al. 1966; Duffy, Teyler et al. 1981; Davis and Squire 1984; Guzowski and McGaugh 1997). D1/D5R blockade by SCH 23390 injections into rats prior to contextual fear conditioning does not affect short term memory but impairs memory consolidation and injection of the D1/D5R antagonist prior to test do not affect the expression of conditioned fear (Inoue, Izumi et al. 2000). Moreover, D1/D5R activation is necessary for the persistence of spatial memory as tested on the Morris water maze (O'Carroll and Morris 2004). As with the necessity of NMDARs in LTP induction and memory acquisition, the D1/D5R is necessary for protein synthesis dependent L-LTP and memory consolidation. Neither the NMDAR nor D1/D5Rs are necessary for the expression of hippocampal dependent memories. The use of pharmacological manipulation has provided great insight into the mechanisms memory acquisition, consolidation and expression. However pharmacological agents produces nonspecific neuronal effects that reduce the precision and accuracy of the aforementioned mechanisms (Mahadevan and Edwards 1991; Tsien, Huerta et al. 1996). Such effects can alter motor performance and place the animal into a noxious state. Additionally, pharmacological agents cannot delineate between D1Rs and D5Rs and thus reduces the precision and accuracy by which the functions of the receptors can be understood in memory consolidation. The advent of genetically manipulated mice ushered in a new era in the study of learning and memory. Genetic techniques have allowed for the specific deletion of single genes

restricting deletion both spatially and temporally bypassing many of the unwanted side affects seen in pharmacological studies.

Genetic Manipulation in the Study of Learning and Memory

Genetic deletion of single genes in mice has provided a clear link between a gene and a behavioral deficit as well as link between deficits in synaptic plasticity in the form of LTP (Silva, Paylor et al. 1992; Silva, Stevens et al. 1992; Tsien, Huerta et al. 1996). The use of genetic techniques addresses two critiques of pharmacological studies: (1) pharmacological agents affect areas beyond the site of injection (2) agonists and antagonists can non-selectively bind to receptors that are not of interest and (3) these agents have nonspecific effects on neuronal activity and molecular mechanisms. The first generation of genetic techniques that were used to connect genes with physiological and behavioral deficits were through the use of global KO mice, where the gene of interest was deleted from the entire genome (Silva, Paylor et al. 1992; Silva, Stevens et al. 1992). Global KOs bypassed the non-specificity of pharmacological agents and thus focused on a single genetic locus and its subsequent protein products. Moreover, global KO mice made the first association between a gene and learning. The first global KO mouse tested on hippocampal dependent learning and memory were mice lacking the αCaMKII gene (αCaMKII -/-). These mice are deficient in hippocampal LTP as well as impaired spatial learning (Silva, Paylor et al. 1992;

Silva, Stevens et al. 1992). In addition the αCaMKII -/- mouse, a mouse lacking the α and δ isoforms of the CREB (CREB -/-) was created. CREB -/- mutant mice, as with the αCaMKII -/- mutants, were deficient in hippocampal LTP and in exhibited deficits in contextual fear conditioning (Bourtchuladze, Frenguelli et al. 1994; Hummler, Cole et al. 1994). As first demonstrated with physiological manipulations, the αCaMKII and CREB global KOs underscore the importance of these proteins in hippocampal synaptic plasticity and learning. Furthermore, global KOs of both the D1R (D1 -/-) and the D5R (D5 -/-) have been created (Drago, Gerfen et al. 1994; Xu, Moratalla et al. 1994; Holmes, Hollon et al. 2001). Pharmacological agents cannot delineate between the D1R and D5R due to high homology in their amino acid sequence. Thus, D1 -/- and D5 -/- mutant mice have allowed for the first line of characterization between these receptor subtypes. CREB integrates the Ca^{2+} and cAMP signal and D1/D5R activation enhances Ca^{2+} and cAMP concentration in postsynaptic neurons. D1 -/- mutant mice exhibit deficits in L-LTP as well as in spatial learning whereas the D5 -/- do not, providing for the first time evidence for functional differences between these two receptor subtypes (Matthies, Becker et al. 1997; Smith, Striplin et al. 1998; El-Ghundi, Fletcher et al. 1999; Holmes, Hollon et al. 2001). Global KO mice have presented a direct connection between a gene, synaptic physiology, learning and memory. However, the primary critique of global KO mice comes from the lack of regional control of genetic manipulation, which can be either deleterious to the animals health or result in nonspecific effects on brain regions

beyond the area of interest. For example, deletion of genes during development could alter brain regions outside the area of interest, which then could impact behavioral or motor performance. Additionally, global deletion of the gene encoding the NR1 subunit of the NMDAR results in neonatal death (Forrest, Yuzaki et al. 1994). Genetic tools became much more powerful with the advent of conditional genetic techniques.

Conditional mutant mice offer spatial control of genetic expression, reducing nonspecific effects observed in global KOs. The non-specific effects of pharmacological agents and global KO mice can be bypassed via the use of the Cre/loxP system that underlies conditional genetic deletion of single genes (Tsien, Huerta et al. 1996; Nakazawa, McHugh et al. 2004). In the Cre/LoxP system two mice are created, the first mouse contains an inserted Cre gene that is expressed by a tissue specific promoter. The second mouse contains two loxP sites that flank the gene (floxed) of interest. The animal produces offspring that are either Cre positive or negative and after successive generations all offspring are homozygously floxed. This system deletes single genes in specific brain region in a spatially controlled manner whereby Cre, a DNA recombines, selectively deletes DNA in the floxed region. As a result, conditional transgenic mice with promoters only active during adulthood can ameliorate consequences of the global KO, such as compensatory upregulation of protein products (Blendy, Kaestner et al. 1996). Many Cre/LoxP transgenic lines have been produced

since their inception in 1996. Mice lacking the NR1 subunit of the NMDAR have been selectively and separately deleted in the DG, area CA1, and CA3 of the hippocampus, bringing even greater specificity to the role of not only the NMDAR in learning and memory but also to the functional role each subregional circuit of the hippocampus (Tsien, Huerta et al. 1996; Nakazawa, Sun et al. 2003; Nakazawa, McHugh et al. 2004; McHugh, Jones et al. 2007). Other conditional KO mice have also been produced, such as mice lacking PKA, which is a target of cAMP and initiates transcription by directly activating CREB, in area CA1 of the hippocampus (Abel, Nguyen et al. 1997). Most recently, transgenic mice that lack the D1R, D5R or both the D1 and D5R have been generated and the characterizations of these mice are the topic of this thesis. The use of global and conditional KO mice has provided a powerful way to study the genetic locus of hippocampal plasticity, learning and memory.

The role of dopamine in hippocampal plasticity, learning and memory is not well understood. Conditional KO mice have greatly advanced the understanding of the role NMDARs play in learning and memory through their spatially restricted deletion. Specifically, NR1 KO studies have directly tested theoretical hypotheses postulated by David Marr over 40 years ago, which could not have been tested without specific genetic manipulation (Marr 1971). The NR1 subunit is not only necessary in LTP induction at the CA3-CA1, mPP-DG synapse but underlies pattern separation functions in the DG, pattern completion and memories of

single experiences in area CA3 and spatial memory acquisition in area CA1 (Marr 1971; McHugh, Blum et al. 1996; Tsien, Huerta et al. 1996; Nakazawa, Quirk et al. 2002; McHugh, Jones et al. 2007). Conditional KO studies, like the mechanical lesions and pharmacological studies that preceded them, have further parsed out the components of memory formation to astonishing degree. Thus, the use of conditional KOs lacking the D1R and D5R will further open up the understanding of how these receptors mediate hippocampal memory formation. Pharmacological studies of dopamine receptors do not delineate between the receptor subtypes that dopamine acts upon resulting in ambiguity of molecular pathways (Missale, Nash et al. 1998). In addition, dopamine receptor subtypes expression overlaps throughout the hippocampus thus preventing pharmacological manipulation from separating the roles of each receptor type at the network level (Fremeau, Duncan et al. 1991; Laplante, Sibley et al. 2004). Global KOs of dopamine receptors have been created, however the lack of spatial and temporal control of genetic deletion does not allow for the direct linkage between the hippocampus with the genetic deletion of dopamine receptors. Moreover, pharmacological and Null KO studies have provided contradictory results (See Chapter 4), which has prevented the progress of our understanding of dopamine's role in hippocampal dependent plasticity, learning and memory. Our lab has generated strains of conditional mutant mice that lack the gene that encodes two dopamine receptor subtypes in the hippocampus. My data clearly shows specific physiological and behavioral phenotypes that have

not been able to be studied by pharmacological manipulation or by global KO mice.

Dopamine Anatomy and Receptor Structure

The ventral tegmental area (VTA) and substantia nigra (SN) are midbrain dopaminergic producing neurons that send efferent's throughout the brain where ~15-18% of the VTA/SN neurons sending inputs to the hippocampus are dopaminergic (Scatton, Simon et al. 1980; Gasbarri, Verney et al. 1994). Projections from the VTA/SN occur in the oriens and molecular layer of the subiculum, all layers of area CA1, stratum oriens of CA3 and the Hilus (Gasbarri, Packard et al. 1994). Increased activity in the VTA/SN increases the DA concentration by transient release the hippocampus (Grace and Bunney 1984). Dopamine (DA) acts upon five distinct forms of dopamine receptors dopamine 1 (D1Rs) through D5 receptors (D5Rs). There are two families of DARs, the D1 like (D1Rs and D5Rs) and the D2 (D2Rs, D3Rs and D4Rs) (Kebabian and Calne 1979; Creese, Sibley et al. 1983). Dopamine receptors are G-protein coupled receptors (GPCRs) and consist of a seven transmembrane domain that interacts with a G-protein (Gingrich and Caron 1993). The D1R has a 466 amino acid (AA) sequence while the D5R has a 475 AA sequence; both D1Rs and D5Rs are intronless and interact with G_s GPCRs. Activation of G_s results in activation of adenylyl cyclase, increasing cAMP concentration leading to CREB mediated

transcription necessary for hippocampal synaptic plasticity, learning and memory. The maximum activation of cAMP via D1R activation is twice as greater as the maximal activation seen with the D5R, however the half maximal effective concentration (EC_{50}) does not differ significantly between the two receptors. No appreciable differences in D1R vs. D5R desensitization have been found (Jarvie, Tiberi et al. 1993). In comparison, the D2 family contains several introns and interacts with G_i GPCRs (Dal Toso, Sommer et al. 1989; Giros, Sokoloff et al. 1989; Grandy, Marchionni et al. 1989; Monsma, McVittie et al. 1989; Selbie, Hayes et al. 1989; Sunahara, Niznik et al. 1990; Grandy, Zhang et al. 1991). Activation of G_i GPCRs inhibits adenylyl cyclase, the G_s and G_i GPCR interaction with adenylyl cyclase functionally categorizes the D1 from the D2 family. The binding results of the D1 family suggest that during bursting activity of VTA neurons, the D1R primarily mediates cAMP activation as compared to D5R contribution. Additionally, when DA concentrations are very low the D5R may be the primarily mediator of cell membrane properties as compared to the D1R. Given the similarity of the D1R and D5R the distribution of these receptors has produced ambiguous results.

The expression pattern of brain D1Rs and D5Rs lacks clarity. The D1 family shares an 80% sequence homology in their transmembrane domains underlying the similarity in their pharmacological profile and the inability of molecular methods in delineating their expression patterns (Sunahara, Niznik et al. 1990;

Sunahara, Guan et al. 1991; Tiberi, Jarvie et al. 1991). D1Rs exhibit the highest expression of all DARs and express primarily in the caudate-putamen, nucleus accumbens, and olfactory tubercle (Fremeau, Duncan et al. 1991; Weiner, Levey et al. 1991). Using in situ hybridization (ISH), it has been demonstrated that within the dorsal hippocampus the D1R expresses exclusively in the dentate gyrus (DG) and area CA2. Posterior to the dorsal region along temporal axis D1Rs are expressed exclusively in the subiculum and at the ventral pole D1Rs express in both area CA2 and the subiculum (Fremeau, Duncan et al. 1991). The precise expression of the D5Rs is occluded by the use of labels that cannot delineate D5Rs from the D1R (Laplante, Sibley et al. 2004). However, expression of the D5R is not ubiquitous as is the D1R, as the D5R shows restricted and localized expression. D5Rs do not express in the caudate-putamen, nucleus accumbens or the olfactory tubercle. D5Rs express most abundantly in the hippocampus and show focal expression in the lateral mammillary nuclei, and the parafascicular nuclei of the thalamus (Tiberi, Jarvie et al. 1991; Gingrich and Caron 1993). D1Rs and D5Rs thus exhibit very little overlap in the rodent brain, the only overlap found is in the DG of the dorsal hippocampus. D1Rs and D5Rs are primarily found post-synaptically, however there is pre-synaptic expression of both D1Rs and D5Rs depending on the brain region they are expressed in. D1Rs are primarily found on postsynaptic spines and D5Rs are primarily found on postsynaptic shafts. Extending in the same direction, regional distribution at the synaptic shows that D1Rs directly interact with the NR1 and NR2B subunits

of the NMDARs (Lee, Xue et al. 2002). Similarly, the D5R directly binds with the γ subunit of the $GABA_AR$ (Liu, Wan et al. 2000).

In addition to the differences in regional expression, it has also been shown that D5Rs have an ~3-10 times greater affinity to DA than the D1R (Sunahara, Guan et al. 1991; Tiberi, Jarvie et al. 1991; Jarvie, Tiberi et al. 1993). Moreover, the D1R is located on chromosome 5 of the mouse while the D5R is located on chromosome 4 (Tiberi, Jarvie et al. 1991). Adding more to the ambiguity of receptor expression comes from D5R pseudogenese that produce mRNA sequences, obscuring again the expression of the functional D5R when probed with RNA sequences (Grandy, Zhang et al. 1991; Weinshank, Adham et al. 1991). Several studies have examined the expression patterns of D1Rs using receptor autoradiography with an antagonist to the D1R family as the probe. At the time the D5R yet been identified and thus the results of D1R expression gave false positives of D1R expression and it was later shown that the probe also binds non-specifically to serotonergic receptors ((Boyson, McGonigle et al. 1986; Dawson, Gehlert et al. 1986; Dubois, Savasta et al. 1986; Savasta, Dubois et al. 1986; Dawson, Barone et al. 1988). These data suggest that although both D1R and D5Rs impinge upon similar molecular pathways, via activation of adenylyl cyclase, their activation profile as well as their expression patterns differ underlying a likely significant difference in physiological and behavioral function. Moreover, pharmacological techniques used to delineate the expression of D1Rs

from D5Rs have only occluded their specific expression patterns, particularly within the hippocampus.

Physiological Studies on D1Rs and D5Rs

Pharmacological studies show the importance of D1Rs and D5Rs in hippocampal plasticity. The dopamine 1 family has been shown to modulate hippocampal synaptic plasticity in both area CA1 and the DG. The D1/D5R antagonist SCH 23390 and the agonist SKF 38393 binds to both D1Rs and D5Rs. D1 family antagonist studies block L-LTP at the CA3-CA1 synapse (Reference). In addition, SCH 23390 blocks E-LTP at the mPP-DG synapse (Reference). Also, SKF 38393 can induce L-LTP at the CA3-CA1 synapse in the absence of strong titanic stimulation, showing DARs are necessary for L-LTP and sufficient with concurrent glutamatergic stimulation. Null KOs of both D1Rs (D1-/-) and the D5Rs (D5-/-) have been produced. In agreement with pharmacological studies D1-/- mice exhibit a deficit in L-LTP at the CA3-CA1 synapse (Reference). No experiments to date have utilized either the D1-/- or the D5-/- in testing L-LTP at the mPP-DG synapse nor has there been a study using the D5-/- in testing L-LTP at the CA3-CA1 synapse. The SCH 23390 CA3-CA1 study and the D1-/- study points to a dilemma in the literature. It has been shown that the D1R expresses in the DG of the dorsal hippocampus but does not express in CA3 or CA1 only in the DG and area CA2 of the dorsal hippocampus. Yet, the D1-/- CA3-CA1 L-LTP

deficit suggests D1R deletion in this region underlies the observed L-LTP deficit. It is likely that nonspecific effects of the D1-/- in other brain regions lead to this observed phenotype. These results point to the incongruent findings between pharmacological and global KO studies and call to attention the lack of clear expression patterns between the two receptor subtypes in current literature. This calls to attention the poor understanding of D1R and D5R functional roles in hippocampal plasticity.

The Role of D1Rs and D5Rs in Hippocampal Learning and Memory

Fear Conditioning and Inhibitory Avoidance

D1Rs and D5Rs modulate hippocampal dependent learning and memory. Subcutaneous injection of SCH 23390 prior contextual fear conditioning reduces freezing when animals are tested 24 hours later. Injection of the antagonist does not affect freezing when animal are tested 5 minutes after training nor is freezing affected when animals receive injection prior to the 24 hour test. These results suggest that D1Rs and D5Rs are necessary for the acquisition or consolidation of contextual fear but they are not necessary for expression. Although contextual fear conditioning is known to primarily rely on the hippocampus the experiment cannot rule out nonspecific effects of the antagonist on other brain regions. In order to directly test the necessity of D1Rs and D5Rs direct injection of SCH

23390 into area CA1 has been used in step down inhibitory avoidance. Injection after training resulted in deficits in the behavioral paradigm when tested 24 hrs later, suggesting that D1R and D5Rs in area CA1 are necessary in step down inhibitory avoidance. Still, pharmacological injections of SCH 23390 cannot delineate D1Rs from D5Rs. D1-/- mice trained in contextual fear conditioning do not show deficits during at a 5 min or a 24-hour test. The ISH data shows that D1R do not express in area CA1 and given that D1-/- mice do not show deficits, the data indicates that D1Rs in area CA1 are not necessary for contextual fear conditioning. However, D5-/- trained on delayed fear conditioning, like the D1-/-, does not show fear conditioning deficits. Behavioral studies utilizing pharmacological lesions or global KOs of the D1Rs and D5Rs again reveal the inability of pharmacology and global deletions to accurately characterize the role of D1Rs and D5Rs in hippocampal dependent learning and memory.

Spatial Learning

The hippocampus is necessary for tasks that rely on spatial navigations, such as the Morris Water Maze (MWM). Mice injected with SCH 23390 show deficits in MWM during training and during the probe trials. Similarly, D1-/- mice show enhanced latency during training and spend significantly less time in the correct quadrant during the probe trial. Additionally, D1-/- are significantly impaired during the reversal phase of MWM training as well as during the probe trial. To

date D5-/- mice have not been tested on MWM. These data suggest that the D1R is necessary in spatial navigation, however, it cannot be concluded that hippocampal D1Rs are necessary for MWM given the global deletion of the D1R. Furthermore, lesions of D1Rs have been shown to increase motor activity in mice, which can affect performance in MWM. These data suggest that spatial learning utilizes D1Rs and perhaps D5Rs in the MWM task.

Conclusions

There is strong evidence that D1Rs and D5Rs are not only important for hippocampal dependent learning and memory but are also functionally distinct and likely subserve specific roles. Physiological studies utilizing pharmacological blockade of D1Rs and D5Rs show that these receptors are important in L-LTP at the CA3-CA1 synapse as well as in E-LTP and the mPP-DG synapse. Additionally, antagonist studies provide evidence that D1Rs and D5Rs are necessary for the acquisition or consolidation, but not expression, of contextual fear conditioning Moreover; the MWM is impaired in animals treated with a D1/D5R antagonist. The pharmacological data provides clear evidence that D1Rs and D5Rs are important in hippocampal protein synthesis dependent plasticity, memory acquisition and/or consolidation. Global KOs of D1Rs or D5Rs also emphasize the importance of D1Rs and D5Rs. D1-/- mice display reduced magnitude of L-LTP at the CA3-CA1 synapse, exhibit intact contextual fear

conditioning and display impaired performance in spatial learning as measured by the MWM task. D5 -/- mice on the other hand do not exhibit LTP deficits at the CA3-CA1 synapse nor do they show deficits in delayed fear conditioning. Pharmacological and global KO studies underline the importance of D1Rs and D5Rs hippocampal plasticity, learning and memory but the differences between pharmacological and genetic studies have yet to be reconciled.

Fundamental issues arise when comparing pharmacological data with global KO studies. First, ISH studies show that the D1R is not expressed in area CA1 of the hippocampus, however D-/- L-LTP experiments show that L-LTP is deficient in these mice. In addition to these data, SCH 23390 studies show deficits in L-LTP at the CA3-CA1 synapse (Fremeau, Duncan et al. 1991). Second, antagonists studies show D1R and D5R blockade hinders acquisition and/or consolidation of contextual fear conditioning. However, D1-/- and D5-/- mice do not show deficits in contextual fear. There are two possible interpretations when analyzing theses data; first, the lack of one member of the D1R family is compensated by the other receptor as both D1Rs and D5Rs increase cAMP concentration. Second, homeostatic effects that are the result of a global KO of the D1R or D5R results in compensatory mechanism that likely rescue the behavioral deficit seen in D1R and D5R pharmacological inactivation. Pharmacological agents cannot distinguish between the D1R and D5R and global KO studies are not region specific. Thus, pharmacological and global KO studies have yet delineated the

functions of D1Rs and D5Rs. In order to distinguish the functions of these two receptors we have generated conditional mutant mice that lack D1Rs and D5Rs, D1Rs, or D5Rs offering both region and receptor subtype specificity. Our data suggests that D1R and D5R deletion impairs memory persistence, that D1R deletion impairment is dependent on the degree of contextual fear training and D5R deletion enhances contextual fear memory

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Chapter 2

Biological and Physiological Confirmation of D1R and D5R Recombination

Abstract

The D1R family has been notoriously difficult to differentiate, resulting in ambiguity of detecting the regional localization of both the D1R and D5R. In order to differentiate D1Rs from D5Rs two individual anti-sense mRNA probes have been constructed. Findings presented here exhibit a unique distribution pattern of the D1R and D5R in the hippocampus, where the D1R and D5R primarily overlap in the dentate gyrus of the hippocampus. Within the hippocampus the D1R is expressed in the DG with some expression also occurring in area CA2, while the D5R is expressed in all hippocampal subregions. In addition, quantified deletion of both the D1R and D5R in each hippocampal subregion is measured, confirming the qualitative *in situ* hybridization data. My findings show that the D1 KO mouse exhibits significant D1R deletion in the DG of the hippocampus and the D5 KO mouse exhibits significant D5R deletion in both area CA1 and the DG of the hippocampus. In order to physiologically verify the KO of the D1R and D5R, L-LTP experiments were conducted at the mPP-DG and CA3-CA1 synapse. The data shows that D1Rs and D5Rs are necessary for the maintenance of L-LTP at the mPP-DG synapse but not at the CA3-CA1 synapse. This later finding is inconsistent with previous pharmacological studies. These data provide the most accurate description of the distribution of D1Rs and D5Rs within the mouse brain to date,

and argues against the necessity of dopamine in the maintenance of L-LTP at the CA3-CA1 synapse.

Introduction

The D1R and D2R families are seven transmembrane domains G-protein coupled receptors that either activate or inhibit adenylyl cyclase, respectively (Kebabian and Calne 1979; Creese, Sibley et al. 1983; Bunzow, Tol et al. 1988; Vallar and Meldolesi 1989; Monsma, Mahan et al. 1990; Zhou, Grandy et al. 1990). The D1R family consists of the D1R and D5R, where the D5R was only recently found as a distinct receptor subtype of the D1 family (Sunahara, Niznik et al. 1990; Grandy, Zhang et al. 1991; Tiberi, Jarvie et al. 1991; Weinshank, Adham et al. 1991). The D1R has been found to express throughout the brain where the highest concentration and distribution is found in the basal ganglia. Other regions with significant expression include the cortex and the limbic regions (Dearry, Gingrich et al. 1990). While the D1R is ubiquitous the D5R has been shown to regionally isolated and difficult to detect due to pseudogenese and similarity to the D1R sequence, however, several regions are thought to express the D5R, which include the striatum, hippocampus and frontal cortex (Grandy, Zhang et al. 1991; Sunahara, Guan et al. 1991). Nevertheless, specific regional expression of the D5R is not widely agreed upon, as past data on the distribution of the D1Rs and D5Rs have been ambiguous due to the similarity of the amino acid sequence of these receptors (Weinshank, Adham et al. 1991; Montague, Striplin et al. 2001; Zelenin, Aperia et al. 2002). Due to limitations of

pharmacological agents that cleanly separate D1Rs from D5Rs, to date there has been minimal progress on separating and identifying D1R distribution versus D5R distribution in the rodent brain.

D1Rs and D5Rs both activate adenylyl cyclase, but to a differing extent and both D1Rs and D5Rs bind to different membrane proteins and have distinct biochemical pathways once activated (Liu, Wan et al. 2000; Sidhu and Niznik 2000; Lee, Xue et al. 2002; Sahu, Tyeryar et al. 2009). Furthermore, D5Rs are ~10 times more sensitive to dopamine than the D1R (Sunahara, Guan et al. 1991). These data coupled with differences in expression patterns suggest significant functional differences at the physiological and behavioral level. In contrast, it has previously been demonstrated that D1/D5R antagonists block L-LTP at the CA3-CA1 synapse and that the D1/D5R agonist can induce L-LTP at the same synapse (Huang and Kandel 1995). The Huang and Kandel study has championed the view that D1R and D5R activation function in an equivalent manner, which has resulted in treating the D1R and D5R as synonymous.

In order to overcome the challenge of differentiating D1R from D5R distribution as well as testing the role of each receptor in underlying synaptic plasticity, we have created probes for D1R and D5R mRNA for *in situ* hybridization experiments and have created three conditional KO mice, the D1 KO, D5 KO and

D1/5 KO. In addition to the conditional KO animals, two global KO mice for the D1R (D1-/-) or D5R (D5 -/-) have been generated, which are utilized to test the accuracy of each mRNA probe. Moreover, these probes are further tested against the conditional KOs. My data shows the D1R and D5R probes specifically bind the D1R and D5R, respectively. In addition, the D1 KO animal exhibits D1R recombination in the DG while the D5 KO exhibits recombination in both area CA1 and the DG. Furthermore, L-LTP experiments at the mPP-DG synapse exhibits deficits in both D1 KO and D1/5 KO mice, however there are no L-LTP deficits observed at the CA3-CA1 synapse in D5 KO animals.

Methods and Materials

Generation of the D1 KO Mouse

The genomic regions containing the D1R gene has been cloned from a C57/Bl6 mouse genomic library (Bacterial artificial chromosome (BAC) library, Genome Systems). The library was screened with a 800bp p^{32} labeled DNA fragment from the 3' region of the mouse C129D1 gene. Two positive BAC clones were obtained. Clone S contains 10kb of genomic sequence upstream of the gene, including the promoter, clone K includes 12kb downstream form the ATG. Both clones were cloned into pBS and mapped. The D1 gene (1.3kb) and part of the promoter were sequenced. A 10kb fragment from clone S, spanning from 7.5kb upstream of the ATG to 2.5kb downstream was inserted into clone K. Then, the Neo/TK/loxP/FRT cassette was cloned into clone K, 4.5kb downstream of the ATG and a third loxP site was introduced into clone S, 5kb upstream from ATG. The DT-A gene was introduced between the downstream arm and the pBS cloning vector. The absence of any ORFs in the genomic regions where DNA fragments were inserted was confirmed. Database searches failed to produce any matches with published coding sequences. The correct sequence and orientation of the D1 gene, the Neor gene, the TK gene and the loxP and FRT sites were verified by sequencing of the replacement vector.

The replacement vectors have been transected into B6 ES cells. Es clones were being selected for Neomycin resistance. Resistant clones will be checked for homologous recombination using 3' and 5' probes located outside the replacement vector. Flp recombinase will then be transiently expressed, eliminating the Neo/tk cassette, leaving one loxP site downstream of the gene of interest to complement the loxP site upstream. Chimeras are generated by blastocycst injection and bred in order to obtain homozygous mutants in, which the targeted gene will be fully functional, but can be excised by Cre recombinase. Homozygous mice will then be crossed to lines expressing Cre. (Credited to Patrik Künzler – obtained from "Progress Report_970107.pdf", 1998)

Generation of the D5 KO Mouse

The genomic region containing the D5R gene has been cloned from female C57/Bl6 mouse genomic library (Stratagene Lambda DASH II Vector, catalog # 945301). The library was screened with a 500 bp p^{32}-labelled DNA fragment, which was obtained via PCR using primers derived from the human D5 sequence. The genomic inserts from seven original positive clones were released from the Lambda vector by digestion with SAL I, and cloned into the DNA plasmid vector pBS (pBluescript II KS^-, Stratagene, GenBank # X52329)

and mapped with several restriction endonucleases. Tow different clones were characterized, together spanning a genomic region flanking the D5R gene over 10kb upstream (clone A) and 12kb downstream (clone B). The D5 gene (1.4kb) was sequenced. A cassette containing a Neomycin resistance gene and a Tyrosine Kinase gene, flanked by a loxP site and an FRT site on each side, was cloned into clone B, 4kb downstream from the ATG. The cassette plus 4kb and 9.5kb, respectively, of genomic DNA downstream of the gene from clone B were then cloned into clone A. A third loxP site was introduced into clone A, 7kb upstream from the gene. The Diphtheria toxin fragment A (DT-A) gene was introduced between the downstream arm and the pBS cloning vector. In the case of homologous recombination f the DT-A gene will be eliminated, whereas it will persist and kill ES clones where the targeting vector has been randomly integrated, enhancing selection from homologously recombinant ES clones. The absence of any ORFs in the genomic regions where DNA fragments were inserted was confirmed by analysis of the DNA sequences obtained from those regions. Database searches (NCBI BLAS search) performed with these sequences failed to produce any matches with published coding sequences. The correct sequence and orientation of the D5 gene, the Neor gene, the TK gene and the loxP and FRT sites, were verified by sequencing of the two replacement vectors. (Credited to Patrik Künzler – obtained from "Progress Report_970107.pdf", 1998)

In situ Hybridization

Brains were removed and frozen fresh in OCT solution. 20 µm parasagital sections were prepared in a cryostat and mounted onto pre-coated glass slides. Sections were post fixed with 4% paraformaldehyde in PBS for 15 min, and treated with 10 µg/ml proteinase K at 37°C for 30 min followed by 0.2 M HCl for 10 min. After rinsing, sections were further incubated in 0.25% acetic anhydride and 0.1 M triethanolamine for 10 min to avoid non-specific binding of the probe. Following dehydration with ethanol, hybridization was performed at 55°C for 18 hours in a hybridization buffer containing 50% formamide. For detection of the mouse D1R or D5R mRNAs, a complementary RNA (cRNA) probe, derived from the AvrII-SphI 0.4-kb antisense DNA fragment of rat D1R or D5R cDNA and, was labeled with [33P]UTP (5x105 cpm), and added to the hybridization buffer. The brain sections were serially washed at 55°C with a set of SSC buffers of decreasing strength, the final strength being 0.2x and then treated with RNase A (12.5 µg/ml) at 37°C for 30 min. The sections were exposed to hyper-beta max for 2 days and were dipped in nuclear emulsion followed by exposure to X-ray film for 2-14 Days. Images were collected with a SPOT camera attached to a microscope. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007).

Quantification of Receptor Deletion

Quantification of receptor deletion was measured from in situ coronal images with measurements obtained from the DG of D1/5 flx (n = 6,2), D1/5 KO (n = 6,2), when quantifying the D1R and D5R mRNA concentration, measurements obtained from area CA1 D1/5 flx (n = 5,2), D1/5 KO (n = 6,2) and measurements obtained from area CA3 D1/5 flx (n = 4,2), D1/5 KO (n = 6,2), when quantifying the D5 mRNA concentration. Images were obtained of coronal slices that had underwent *in situ* hybridization, see aforementioned paragraph. Receptor deletion was quantified by measuring the change of the grayscale gradient of imagines these images. The 16-bit grayscale value range is between 65536 (pure black) to 0 (pure white). High values relate to a higher concentration of ligand binding to the receptor while low high values relate to little to no ligand binding to the receptor. Intermediate values relate to non-saturated binding of the ligand to the receptor. Area CA1 and the DG are quantified by the grayscale values described above using the software application "ImageJ". The area of interest (i.e. the DG) is inscribed and the mean grayscale value is calculated. The mean grayscale value depicts the concentration of the receptor. These values are normalized to dorsal thalamus where there is very little to no expression of the D1R or D5R.

Slice Physiology

Hippocampal slices were taken from, 30-40 week old, male mice from both the D5 flx and D5 KO line. Animals were sacrificed by cervical dislocation and immediately decapitated. The brains were removed and placed into a cold ACSF solution. Transverse slices were obtained at 400 uM increments using a manual tissue chopper. Slices were incubated for 2 hours in an interface chamber and were continuously perfused with aCSF at 32 ^{o}C.

All recordings were conduced at 32 ^{o}C in an interface chamber. Bipolar, steel, stimulating electrodes were placed onto the hippocampal slice within the stratum radiatum (SR) in area CA1 in order to stimulate the Schaffer Collateral (SC) pathway. Extracellular glass microelectrodes filled with aCSF were placed in the SR in order to measured the fEPSP elicited by SC stimulation. Slices were texted for maximal EPSP amplitude and only slices with an amplitude above 4 mV were used for experimentation. For baseline recordings and post tetanus test pulses, the stimulation strength was adjusted to ~40% of the maximum amplitude.

In order to test input specificity, two stimulating electrodes were placed in the SR, with one electrode towards the border of the CA1-CA3 region and the second towards the border of CA1 and the subiculum. This was done to ensure activation of separate SC fibers. After the maximum amplitude of the EPSP was obtained, the stimulus intensity was readjusted to ~40% of maximum EPSP amplitude. Pathway independence was confirmed by the lack of paired-pulse facilitation when stimulation pulses were delivered from one pathway followed by a second stimulation delivered to the second pathway with stimulation intervals of (in mS): 500, 50, 75, 100, 150, and 200. Approximately 10 seconds was given between each pair of test pulses.

In order to test the basal transmission and maximum EPSP amplitude of synaptic output, the SC were stimulated at increasing current amplitudes, producing an input-output (IO) curve. An IO curve was given for each independent pathway, each pathway was given a current pulse that began at 0.01mA and increased to 0.1mA at increments of 0.01mA, this was followed by a current pulse at 0.12mA and at 0.15mA. Each pathway input-output curve was conducted independently. D5 KO (n = 14), D5 flx (n = 30).

Paired pulse facilitation was conducted in order to test presynaptic integrity. After the maximum amplitude of the EPSP was obtained, the stimulus intensity was

readjusted to ~40% of maximum EPSP amplitude. Two stimulating electrodes were placed in the stratum radiatum, with one electrode towards the border of the CA1-CA3 region and the second towards the border of CA1 and the subiculum. Each pathway was given a paired pulse facilitation protocol. A paired current pulse current was given at the following delays (in mS): 50, 75, 100, 150, 200 and 500. Approximately 10 seconds was allowed for between each test pulse. D5 KO (n = 31), D5 flx (n = 21).

Potentiation was induced after a stable 35 minute baseline was obtained for both SCH and vehicle experiments. In experiments where no SCH was used, an L-LTP protocol was given after a 20 min stable baseline. A single test pulse was given every 60 seconds before and after the potentiation protocol. Three trains of 100 pulses at 100Hz, with an intertrain interval of 10 min, induced robust potentiation at the CA3-CA1 synapse, which lasted for at least 3 hours. The amplitude of the pulse train was set to ~40% of the maximum EPSP amplitude obtained from the IO curve. In the SCH 23390 experiments, the compound was diluted to 0.1uM and (or vehicle) was delivered 10 min after a stable baseline was obtained. SCH was discontinued after the last tetanus stimulus was given. D5 KO (n = 4), D5 flx (n = 4).

In vivo Physiology

D1 flx, D1 KO, D1/5 flx and D/5 KO mice between the ages of 30 – 40 weeks underwent mPP-DG L-LTP experiments (D1 KO, n = 7; D1 flx, n = 4; D1/5 KO, n = 9; D1/5 flx, n = 6). Mice were placed into a chamber and sedated with isoflurane for approximately 20 seconds in order to place the animal into the stereotaxic instrument. Mice continued to receive isoflurane during the duration of recordings. A rectal thermometer was used to maintain the animal at 37°C using a heating blanket. Two holes were made using a dental drill with the recording electrode placed into the hilus of the dentate gyrus (2mm posterior from bregma and 1.5mm lateral to the midline) and the stimulating electrode placed into the mPP (3mm lateral from lambda) ipsilaterally to the recording electrode. Each electrode lowered to approximately 1.5mm from the brain surface. Recordings primarily occurred in the right hemisphere, however, when responses in the right hemisphere were not strong enough the recording and stimulating electrodes were placed in the left hemisphere.

In each experiment an input-output curve was assessed from 0 uA through 460 uA with 20uA steps (D1 KO, n = 8; D1 flx, n = 5; D1/5 KO, n = 9; D1/5 flx, n = 6). Three recordings were taken at each step and averaged. Test pulses for the L-LTP experiments were set to 40-60% maximum value of the EPSP amplitude,

where a population spike of at least 5 mV was induced. L-LTP was induced by a theta burst protocol where 6 trains were given at 5 Hz where each train consisted of 6 pulses at 400 Hz, this was repeated 6 times with a 30 second interval.

Results

D1R Deletion Occurs in the DG while D5Rs Deletion Occurs in the DG and Area CA1 of the Hippocampus

D1 KO, D5 KO and D1/5 KO mice were generated by crossing the CaMKII-Cre mouse line with floxed (fD1, fD5, fD1/5) mice. In addition to conditional KO mice, mice with global deletions of D1R and D5R were also generated. Two probes were constructed in order to separately identify the D1R and D5R for *in situ* hybridization studies (Fig 2-1a,b). *In situ* data for the D1 -/- shows no signal in coronal slices (Fig. 2-2a) when tested against the D1R mRNA probe. The D1 -/- line does show a signal when tested against the D5R mRNA probe (Fig. 2-2b). Likewise *in situ* data for the D5 -/- shows no D5R mRNA signal in coronal slices (Fig. 2-3a), although there is a prominent D1R mRNA signal in the D5 -/- line (Fig. 2-3b). Each probe was tested against the D1/D5 KO animal. The D1 receptor exhibits full recombination by 20 weeks of age, while the D5 receptor exhibits recombination by 25 weeks and shows full receptor deletion by 30 weeks (Fig. 2-4 and 5).

In addition to validating recombination of the D1R and D5R in coronal slices, sagittal sections were also utilized in order to observe the rostral-caudal deletion of the D1R and D5R. The D1R exhibits strong expression in the caudate-putamen as well as in the olfactory tubercle (Fig. 2-6a). The D1 KO animal shows recombination in the caudal portion of the caudate putamen as well as recombination in the olfactory tubercle (Fig. 2-6b). The D5R does not exhibit expression outside the hippocampus and D5R recombination in the D5 KO is limited to area CA1 and the dentate gyrus, which is in agreement with the coronal *in situ* data (Fig. 2-7 and 5).

In order to validate the qualitative *in situ* hybridization data, I quantified the expression of both the D1R and D5R. The D1R primarily expresses in the DG of the hippocampus, thus the positive D1R signal was quantified in the flx animal and compared to that of the D1 KO animal D1R signal. Figure 2-8a ($p < 5 \times 10^{-8}$), compare to flx, Student's t test) shows that D1R deletion is significantly and greatly reduced in the dentate gyrus of the D1 KO animal. The D5R is significantly and greatly reduced in area CA1 and in the dentate gyrus, but there is no significant difference in area CA3 of the D5 KO animal (Fig. 2-8b; $p < 7 \times 10^{-5}$, c; $p > 0.40$, d; $p < 5 \times 10^{-8}$, compare to flx, Student's t test). These data confirm the qualitative date provided by *in situ* hybridization, where the D1R is deleted in the dentate of the D1 KO animal and the D5R is deleted in area CA1 and dentate, but not in area CA3 of the hippocampus.

CA3-CA1 L-LTP is Intact in D5 KO Mice

We examined ex-vivo synaptic transmission and plasticity at the CA3-CA1 synapse in D5 KO mice and control littermates between 30 and 40 weeks of age. D1 KO animals were not tested due to the lack of expression of the D1R in area CA3 and CA1 in the hippocampus (Fig. 2-4). An input-output curve was generated by stimulation of the CA3 SC's with the fEPSP was measured in area CA1, which did not show significant differences between D5 KO and control littermates (Fig. 2-9a). In addition to the input-output curve, pair-pulse facilitation was measured as an index of presynaptic function; no significant differences were found between genotypes (Fig. 2-9b). Next, L-LTP was induced by a three train, 100Hz tetanus protocol, which has previously been shown to be dopamine dependent (Huang and Kandel 1995; Navakkode, Sajikumar et al. 2007). We found robust potentiation in D5 KO mice and control littermates, suggesting D5Rs are not necessary for this potentiation protocol. In order to test if residual D5R activation was sufficient or if immeasurable D1Rs mediated the observed L-LTP, the D1/5R antagonist, SCH 23390, was applied to the hippocampal bath. Using the same potentiation protocol as above, we found robust potentiation in both the D5 KO and the control littermates in the presence of SCH 23390, there was no significant between genotypes (Fig. 2-10). Our results show that the three-train tetanus protocol is not D1R or D5R dependent.

D1/D5 KO Mice Exhibit L-LTP Deficits at the mPP-DG Synapse

Both D1Rs and D5Rs express in the DG and D1/D5R antagonist experiments have shown that these receptors are necessary to induce E-LTP at this synapse (Kusuki, Imahori et al. 1997). An input-output curve was generated by stimulation of the mPP and the fEPSP was measured in the dentate hilus of the hippocampus. The input-output curve of the D1 KO and D1/5 KO mice do not exhibit significant differences between littermate controls (Fig. 2-11a and 12a, respectively). When a theta-burst stimulation protocol was given at the mPP there was significant potentiation of the mPP-DG synapse. The slope of the fEPSP of the D1 KO mice returns to baseline within one hour (Fig. 2-11b). Additionally, there is a significant deficit in the late-phase magnitude of potentiation in D1/D5R KO mice (Fig. 2-12b; $p < 0.05$, average at 175-180 min time point, compare to flx, Student's t test). The fEPSP at the mPP-DG synapse in the D1/D5 KO mice returned to baseline post induction within 90minutes and the control mice show sustained L-LTP (Fig. 12b). This is in agreement with pharmacological studies that block both the D1 and D5R.

Discussion

The D1R is primarily expressed in the DG of the hippocampus, while the D5R is expressed in all hippocampal subregions (Fig. 2-3 and 4). We show a unique distribution of the D1R and D5R with the primary overlap of these receptors occurring in the DG, a novel finding. These findings further extend the precise distribution profile of each receptor in that we are able to differentiate each receptor subtype with greater accuracy due to the subregion specific deletion of each receptor subtype

These data show that CA3-CA1 L-LTP is not dependent the D1R family (Fig. 2-10). The tetanus protocol in this study has been used by several groups that show the necessity of D1/5Rs in L-LTP (Frey, Schroeder et al. 1990; Huang and Kandel 1995). However, these studies were done in rats, which may be one reason for these observed differences. Moreover, D1/5R antagonists have been shown to bind and activate to serotonergic receptors as well as alter LTP in area CA1. This suggests that deficits seen in SCH pharmacological studies may be due to that activity of serotonergic receptors (Hicks, Schoemaker et al. 1984; Bischoff, Heinrich et al. 1986; Briggs, Pollock et al. 1991; Woodward, Panicker et al. 1992; Shakesby, Anwyl et al. 2002; Kojima, Matsumoto et al. 2003; Ryan, Anwyl et al. 2008; Zarrindast, Honardar et al. 2011).

Our *in situ* data may not be able to detect fine distribution of D1R expression within the CA1 pyramidal layer. For example, it has been reported that the D1R exhibits expression in the stratum lacunosum moleculareinthe distal dendrites of CA1 pyramidal neurons (Lisman and Grace 2005). Still, we conclude that D1Rs are not necessary for L-LTP at the CA3-CA1 synapse due to the robust L-LTP observed in D1/5 KO animals (data not shown) and the robust L-LTP observed in D5 KO animals with and without SCH (Fig. 2-10).

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Figure 2-1

D1R Probe mRNA Nucelotide Sequence

a

5' – ACAAAAGCACAATGGTGTTCCATCAGGAGCATCTCCATAGCAATCCAAGCCATACCA
GGAAGAGAGCCGCTTGCTTTCCACCTGTCTTCTGGGTTCAGTGCTCCAGGTCGCTGTTCC
CTGGCATCCGCTGGTCCCTAGATTCCCCAAGGAATGCATAGGCTTTTAAGCATACTCTAA
GAGTCTGGGGCCTCTTCCTGGTCAATCTCAGTCACTTTTGGGGATGCTGCCTCTTCTTCT
GAGACACAGCCTAAAATACATGCATTTCTCCTTCAAGCCCCTGGTGCCACATCTCTCCAA
ATGCC – 3'

D5R Probe mRNA Nucelotide Sequence

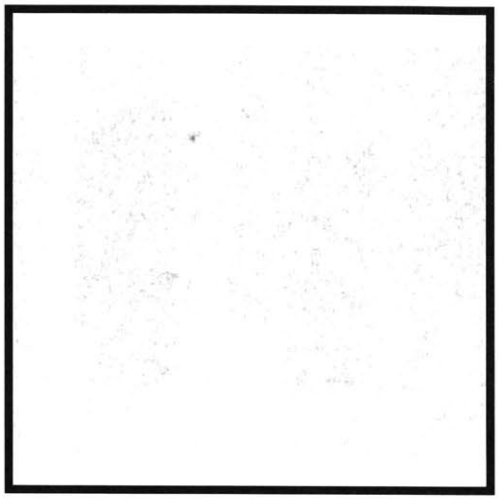
b

5' – CCAAAATCCTGCTGTCTTCCAAGAGCACTGGCACTTGTGGTTTCTCTAGGAGAAACA
CTGAGCACCAACTGGCAAAGCAAAGGTGACTGCCCCTCCTCCCAGCCACAAATGAATGTA
CTGTGCGCTTATGGAAACCACAACAAATCAGGGAGAAATCCCGGCCACAGGAAAGACCCT
TCAACCTGCACTAAAGCAGCAGCCCGAGAACAGGGGGCTATGGTCCCAAAGTCTAGAAAG
TCACAGACCATACCAGCAATTGCCACTCAGACCTGTCATTTAAAAAGCAACCCAGGTGCAA
GTCACAGAACAAGCCTCTGTTAGAAAGGGTAAATTGAGGTGTACTTCTTAAAGGACCAGGT
TCCACTTTCTCGTCTCTAAAGGGAACTCT – 3'

Figure 2-2

D1 -/- *In Situ* Hybridization for the D1R and D5R mRNA Probe

a **D1 mRNA Probe**



b **D5 mRNA Probe**

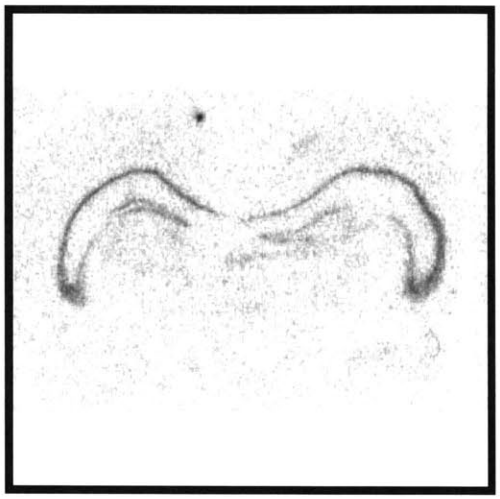
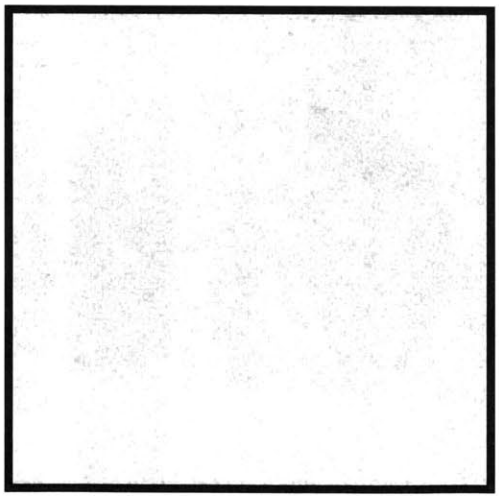


Figure 2-3

D5 -/- *In Situ* Hybridization for the D1R and D5R mRNA Probe

a **D5 mRNA Probe**



b **D1 mRNA Probe**

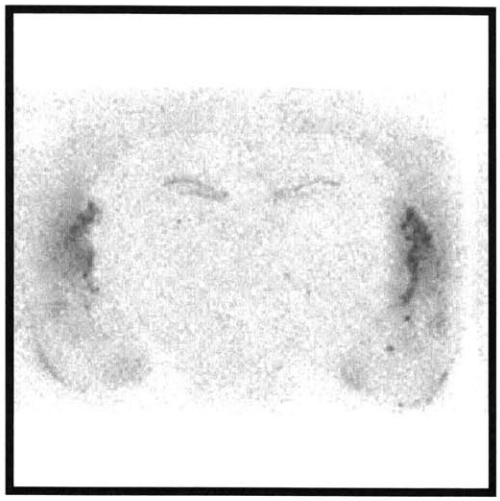


Figure 2-4

Developmental Timeline of D1R Recombination

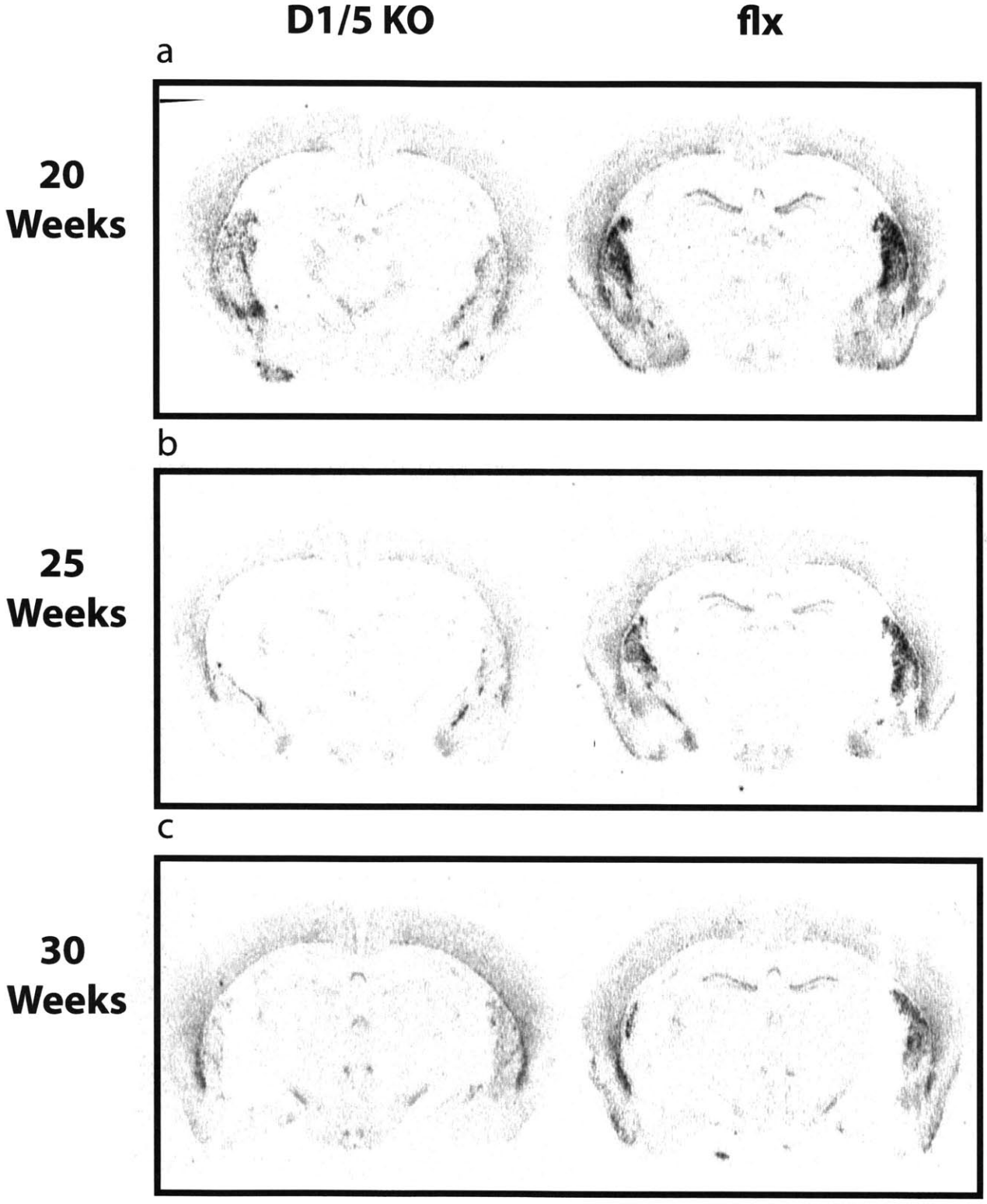


Figure 2-5

Developmental Timeline D5R Recombination

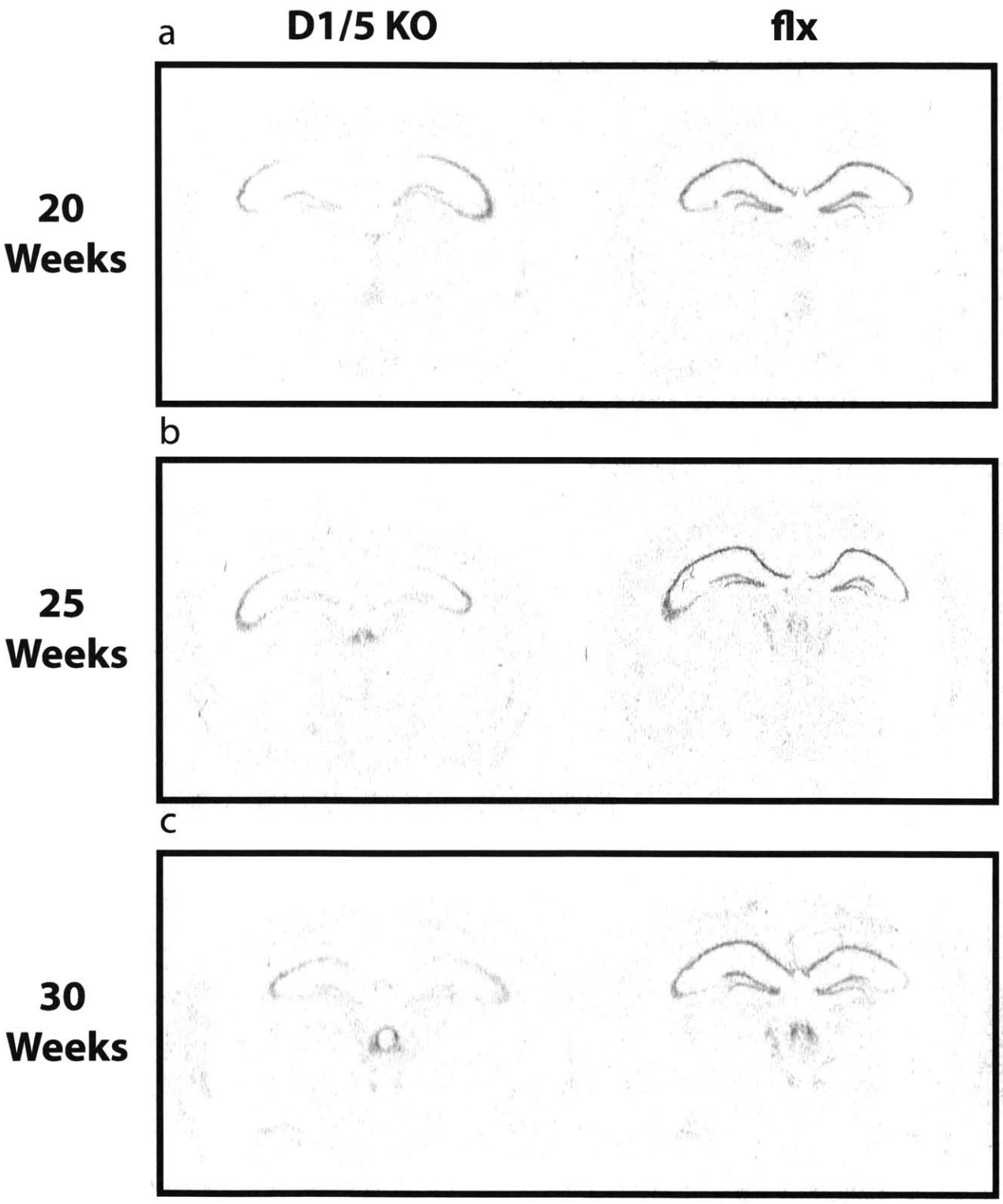
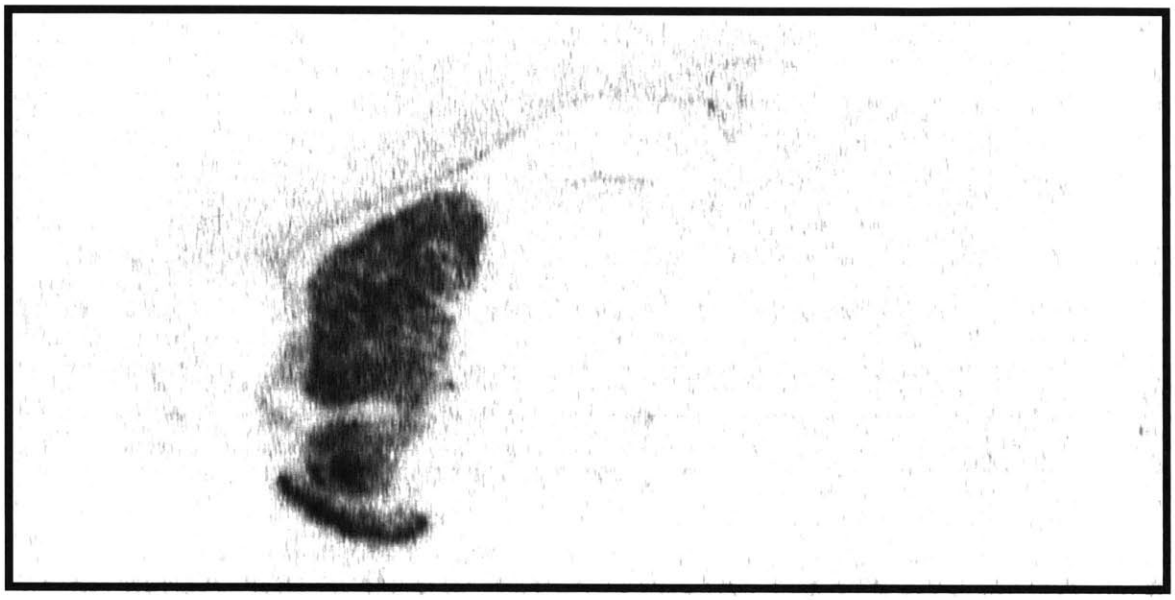


Figure 2-6

In Situ Hybridization - Saggital Section
D1R Expression

a **flx**



b **D1/5 KO**

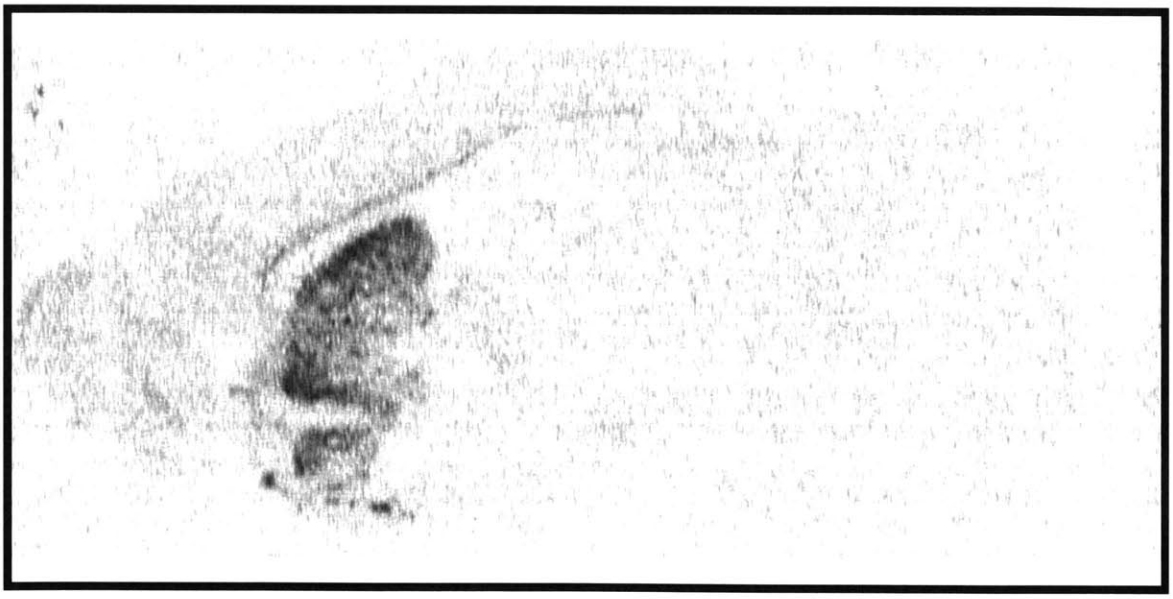
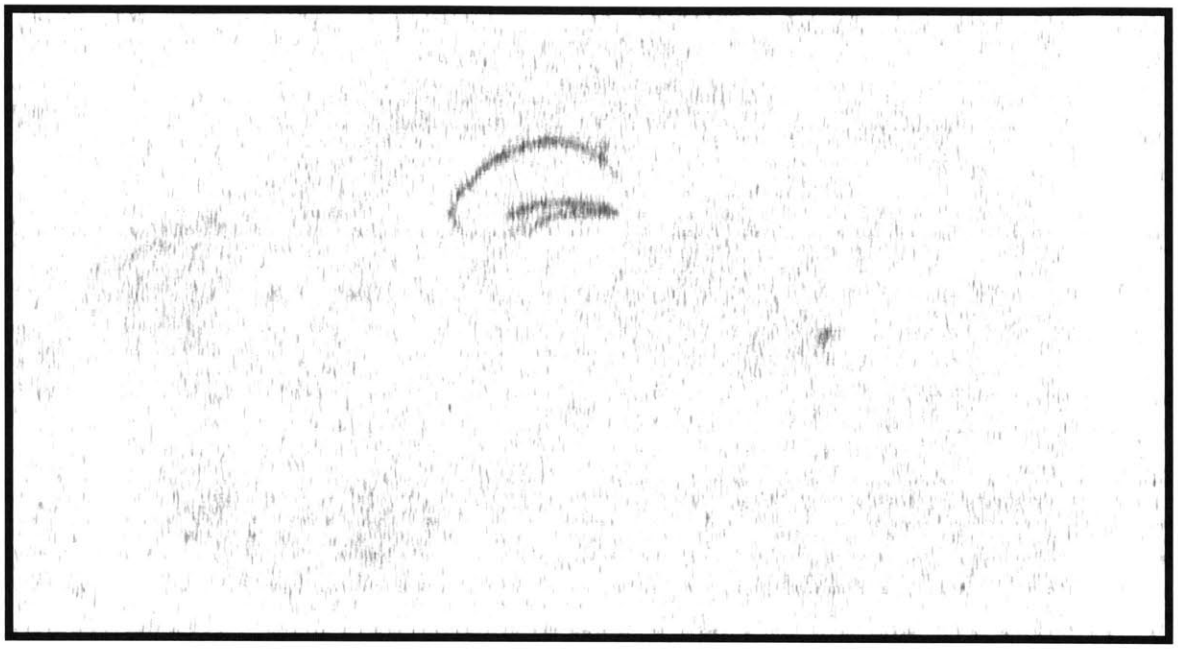


Figure 2-7

In Situ Hybridization - Saggital Section
D5R Expression

a **flx**



b **D1/5 KO**

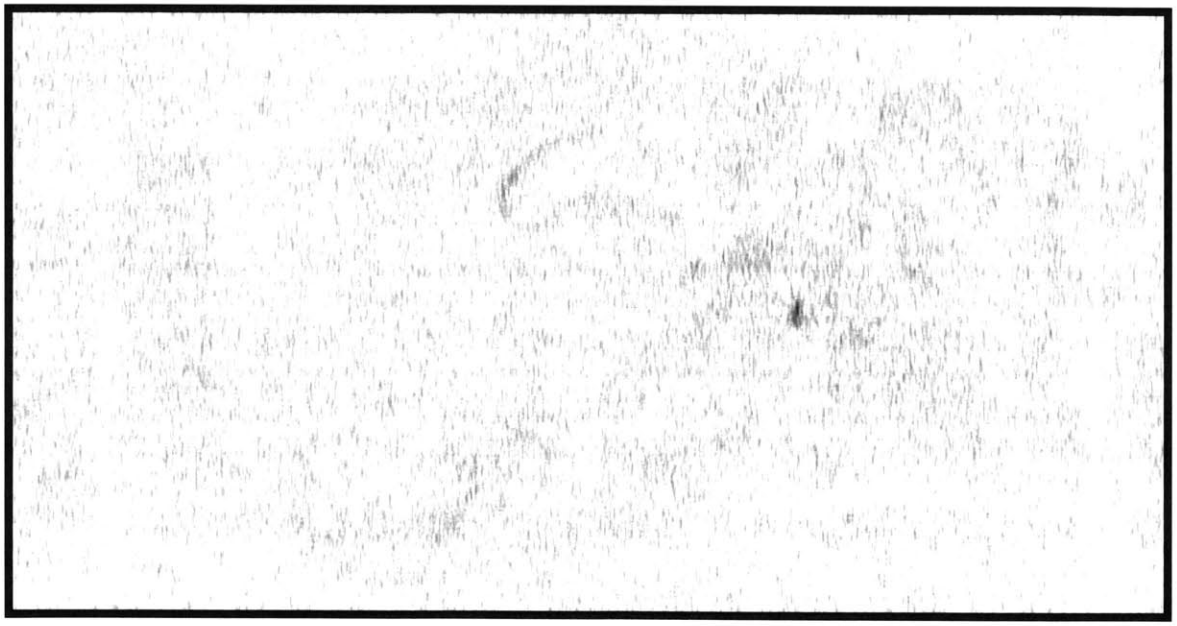


Figure 2-8

Quantification of *In Situ* Hybridization Data

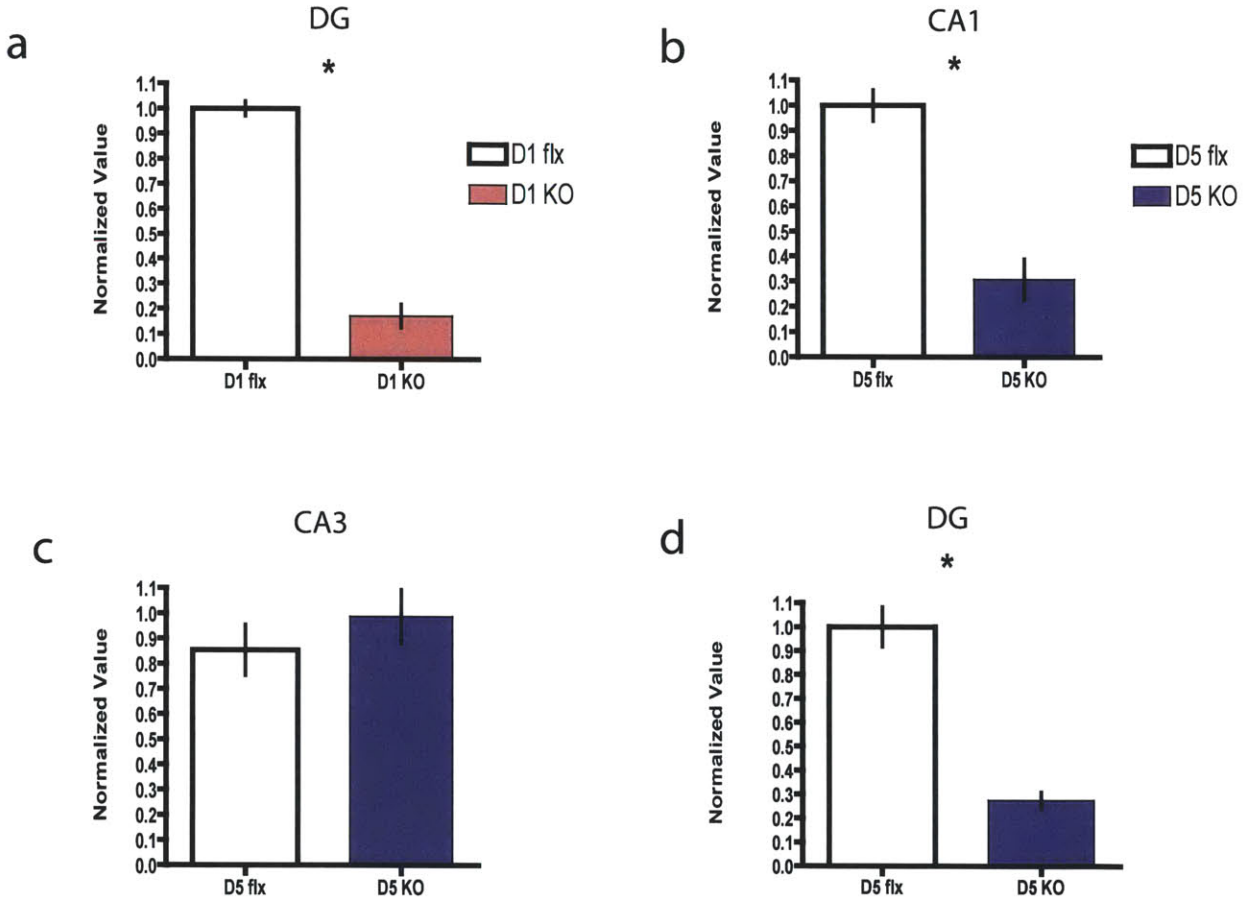
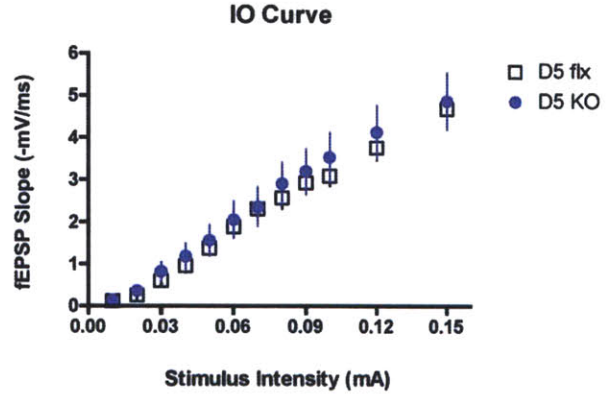


Figure 2-9

Ex Vivo CA3-CA1 IO Curve and PPF

a



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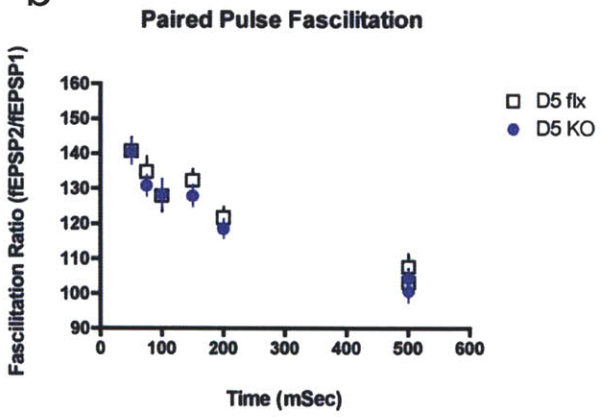
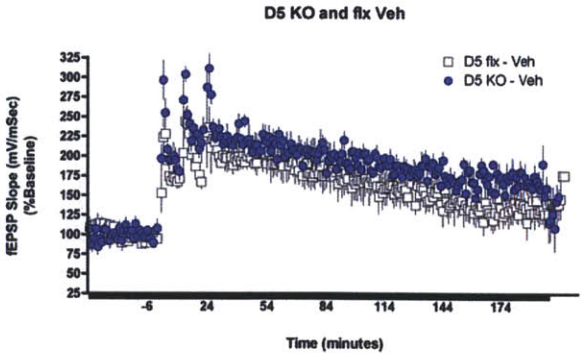


Figure 2-10

Ex Vivo CA3-CA1 L-LTP in D5 KO Mice Veh and SCH 23390

a



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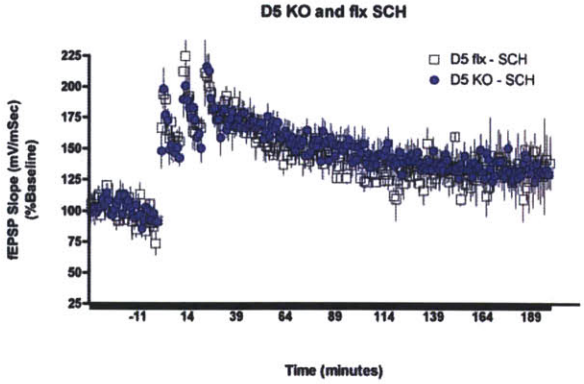
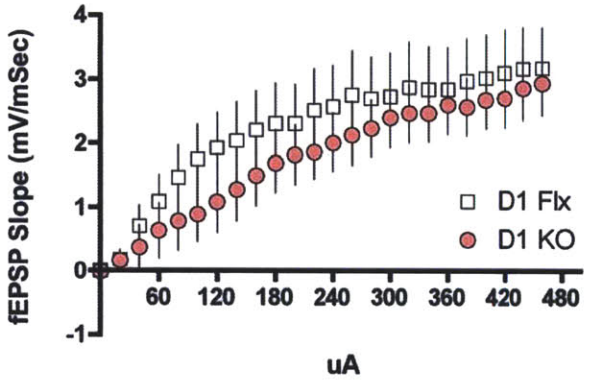


Figure 2-11

In Vivo mPP-DG IO Curve and L-LTP - D1 Line

a



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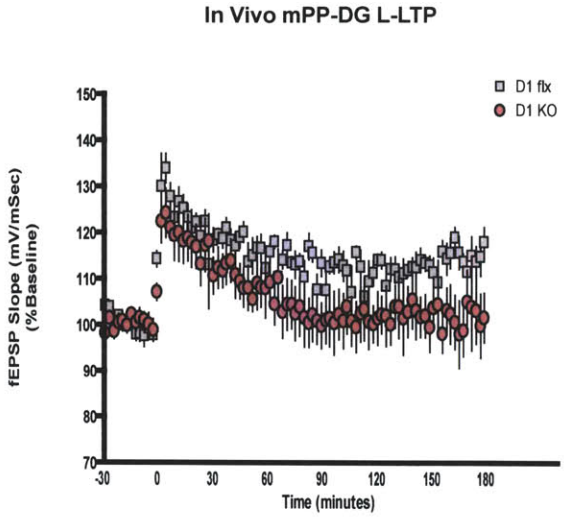
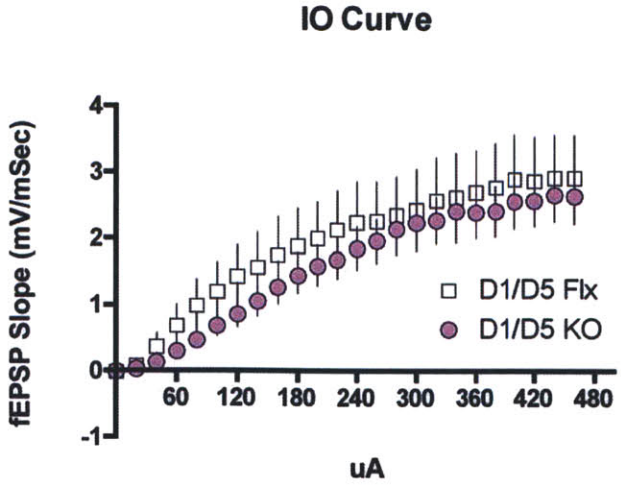


Figure 2-12

In Vivo mPP-DG IO Curve and L-LTP - D1/5 Line

a



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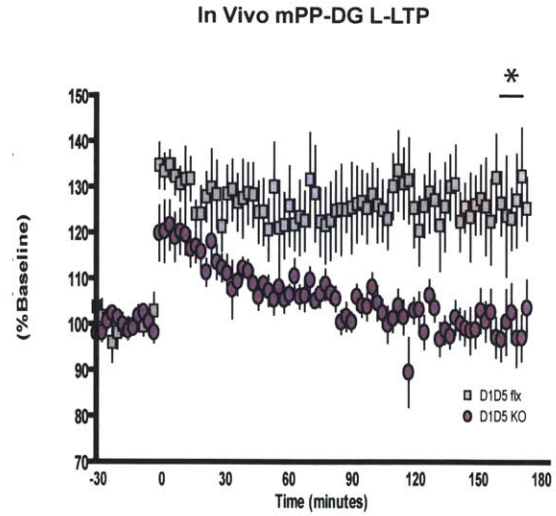

Figure 2-1. D1R and D5R Probe mRNA Nucleotide Sequence.

(a) D1R mRNA nucleotide sequence for in situ hybridization. The mRNA region is found on chromosome 4 of the mouse genome and pertains to a non-coding region near the N-terminus domain of the protein.

(b) D5R mRNA nucleotide sequence for in situ hybridization. The mRNA region is found on chromosome 5 of the mouse genome and pertains to a similar region of the D1R mRNA nucleotide sequence.

Figure 2-2. D1-/- In Situ Hybridization for the D1R and D5R mRNA Probe.

(a) No D1R mRNA signal is observable in the D1 -/- mouse. Coronal slice , bregma ~2.0mm.

(b) A strong D5R mRNA signal is observable in all hippocampal subregions of the D1 -/- mouse. Coronal slice, bregma ~2.5mm.

Figure 2-3. D5-/- In Situ Hybridization for the D1R and D5R mRNA Probe.

(a) No D5R mRNA signal is observable in the D5 -/- mouse. ~ Bregma -2.0mm

(b) D1R mRA probe signal is present in the DG of the hippocampus and the caudate-putaman of the D5 -/- mouse. Coronal slice ~ Bregma -2.0mm

Figure 2-4. Developmental Timeline of D1R Recombination

(a) 20 week time point – Full D1R recombination in the DG occurs by 20 weeks.

(b) 25 week time point - D1R shows further recombination in the basal ganglia.

(c) 30 week time point - D1R recombination exhibits no difference by 30 weeks in comparison to 25 weeks.

Figure 2-5. Developmental Timeline of D5R Recombination

(a) 20 week time point - D5R recombination is observable by 20 weeks in area CA1 of the hippocampus with weak recombination in the DG.

(b) 25 week time point - D5R shows further deletion in CA1 and DG.

(c) 30 week time point - D5R recombination exhibits further recombination in area CA1 and dentate gyrus.

Figure 2-6. In Situ Hybridization – Sagittal Section D1R Expression

(a) D1/5 flx mouse – D1R mRNA exhibits strong expression in the caudate-putaman as well as in the dentate gyrus of the hippocampus and cortical layer 6b.

(b) D1/5 KO mouse – exhibits decreased D1R mRNA signal in the caudal caudate-putaman and full recombination in the DG.

Figure 2-7. In Situ Hybridization – Sagittal Section D1R Expression

(a) D1/5 flx mouse – D5 mRNA expression occurs in all subregions of the hippocampus.

(b) D1/5 KO mouse – the D5R undergoes full recombination in area CA1 and DG of the hippocampus.

Figure 2-8. Quantification of In Situ Hybridization Data

(a) D1/5 KO mice (n = 6,2) exhibit significantly reduced ($p < 5 \times 10^{-8}$) D1R mRNA signal in the DG of the hippocampus as compared to D1/5 flx mice (n = 6,2).

(b) D1/5 KO mice (n = 6,2) exhibit significantly reduced ($p < 7 \times 10^{-5}$) D5R mRNA signal in area CA1 of the hippocampus as compared to D1/5 flx mice (n = 5,2).

(c) D1/5 KO mice (n = 6,2) do not exhibit reduced D5R mRNA signal in area CA3 of the hippocampus as compared to D1/5 flx mice (n = 4,2).

(d) D1/5 KO mice (n = 6,2) exhibit significantly reduced ($p < 5 \times 10^{-8}$) D5R mRNA signal in the DG of the hippocampus as compared to D1/5 flx mice (n = 5,2).

Figure 2-9. Ex Vivo CA3-CA1 IO Curve and PPF

(a) D5 KO (n = 14) and flx mice (n = 30) exhibit similar IO curves across increasing stimulus intensity.

(b) D5 KO (n = 31) and flx mice (n = 21) exhibit similar paired pulse facilitation curves across increasing inter-stimulus intervals

Figure 2-10. Ex Vivo CA3-CA1 L-LTP in D5 KO Mice, Veh and SCH 23390

(a) Vehicle group. A three-tetanus L-LTP protocol at the CA3-CA1 synapse. D5 KO mice (n = 4) do not exhibit significant differences in L-LTP magnitude across the time course of the experiment as compared to D5 flx mice (n = 4).

(b) SCH 23390 group. A three-tetanus L-LTP protocol at the CA3-CA1 synapse in the presence of SCH 23390. D5 KO mice (n=6) do not exhibit significant differences in L-LTP magnitude across the time course of the experiment as compared to D5 flx mice (n=3).

Figure 2-11. In Vivo mPP-DG IO Curve and L-LTP – D1 Line

(a) D1 KO (n = 8) and flx mice (n = 5) exhibit similar IO curves across increasing stimulus intensity.

(b) A theta burst L-LTP protocol is given at the mPP-DG synapse. D1 KO mice (n = 7) do not exhibit significant differences in L-LTP magnitude across the time course of the experiment as compared to D1 flx mice (n = 4).

Figure 2-12. In Vivo mPP-DG IO Curve and L-LTP – D1/5 Line

(a) D1/5 KO (n = 9) and flx mice (n = 6) exhibit similar IO curves across increasing stimulus intensity

(b) A theta burst L-LTP protocol is given at the mPP-DG synapse. D1/5 KO mice (n = 9) exhibit significant reduction in L-LTP magnitude ($p < 0.05$, average at 175-180 min time point) as compared to D1 flx mice (n = 6).

Data are presented as mean +/- SEM.

Chapter 3

Behavioral Batteries and Test of Amygdala Integrity

Abstract

Contextual fear conditioning and the Morris water maze are two hippocampal dependent paradigms. Contextual fear requires that animals are not pre-disposed to anxiety and require that mice do not display differences in the sensitivity to pain. Additionally, any deficits that occur with contextual fear conditioning could be due to amygdalar impairment, thus it is necessary to test the functional capabilities of the amygdala. Similarly, in order to acquire spatial learning via water maze, it is necessary to have intact motor capabilities. D1 KO, D5 KO and D1/5 KO mice are trained on both of the aforementioned paradigms (See Chapters 4-6); however the behavioral phenotypes these mice display may not be due to hippocampal-mediated deficits. Therefore mice are tested on a set of behavioral batteries that analyze the sensitivity of pain, motor locomotion, anxiety and amygdala-based deficits. There are no significant differences in these KO mice when given a pain sensitivity test, a rotarod motor test, an anxiety test on the elevated plus maze, the open field test, and do not show deficits in a delayed fear-conditioning paradigm. This suggests the KO lines do not suffer from gross motor deficits, general anxiety and have an intact amygdalar processing given these specific behavioral protocols.

Introduction

Pain Sensitivity, Anxiety and Motor Locomotion

D1Rs are highly expressed in the basal ganglia, a structure that underlies motor control. Deletion of D1Rs in the basal ganglia can lead to altered motor capabilities (Xu, Moratalla et al. 1994). D1/5 KO mice show deletion of the D1R in the caudal basal ganglia, which has the potential to alter motor capabilities in these mice (Fig. 2-6 and 7). Contextual fear conditioning is assessed by freezing and the Morris water maze by directed swimming. These measures rely on the functions of many brain regions that are sensitive to different behavioral outputs other than learning. In order to measure the degree to which the KO's phenotypes are unrelated to learning in fear conditioning and water maze, we looked at differences in pain gross motor activity, pain sensitivity, and general anxiety.

Delayed Tone Fear Conditioning

The hippocampus and amygdala underlie the acquisition of tone fear conditioning (Phillips and LeDoux 1992). Tone fear conditioning utilizes a neutral auditory cue, the conditional stimulus that predicts an oncoming foot shock, and during training an association between the tone and to the unconditional stimulus occurs

(foot shock). The cue may either co-terminate with the shock; known as delayed fear conditioning, or a trace interval may be placed between the cue termination and initiation of the foot shock, known as trace fear conditioning. Delayed fear conditioning engages both the hippocampus and amygdala, however the tone functions as a powerful predictor to the shock as compared to the contextual arena (Phillips and LeDoux 1992). Thus, in delayed fear conditioning the amygdala bases the majority of the fear response (Kim and Fanselow 1992). Delayed fear conditioning can be acquired without an intact hippocampus and lesions to the amygdala prevents acquisition of delayed fear conditioning, additionally, retrograde amnesia occurs with post training amygdalar lesions (Kim and Fanselow 1992). Trace fear conditioning primarily engages the hippocampus, lesions of the hippocampus prior to trace conditioning prevents acquisition while post training lesions of the hippocampus results in retrograde amnesia (Huerta, Sun et al. 2000). Lesions of the amygdala do not significantly impair trace fear conditioning (Raybuck and Lattal 2011).

Delayed fear-conditioning tests the integrity of amygdala (Phillips and LeDoux 1992). To determine if the D1, D5 or D1/5 KO interferes with contextual fear conditioning, which is classically viewed as being hippocampal-dependent, we also tested our mice in delay fear conditioning (Phillips and LeDoux 1992). Delay fear conditioning engages many of the same brain functions as contextual fear conditioning, such as the hippocampus and in some cases the striatum. Delay fear conditioning differs in the nature of the conditioned stimulus in comparison to

contextual fear conditioning, where the former utilizes discreet stimuli (such as a tone) and the later utilizing multimodal cues that associate to the unconditioned stimulus. Binding multimodal cues into a contextual representation is a reason why contextual fear conditioning relies on an intact hippocampus (and amygdala), while delay conditioning does not require an intact hippocampus (Phillips and LeDoux 1992). Testing our mice in both delay and contextual conditioning gives us insight into whether observed learning phenotypes are specific to hippocampal-dependent tasks (as hippocampal lesioning studies suggest), or whether manipulation of dopamine in the hippocampus can influence learning more generally, even learning of tasks that do not require an intact hippocampus, such as delayed fear conditioning.

Methods and Materials

Pain Sensitivity

Mice are placed on a 12 hr light/dark cycle with 2-4 animals/cage and separated by sex; only males are used for behavioral experiments. All experimenters are blind to the genotype at the time of training and analysis. D1 flx (n = 9), D1 KO (n = 8), D5 flx (n = 9), D5 KO (n = 7), and D1/5 flx (n = 10), D1/5 KO (n = 7) mice are run at 30-40 weeks of age. A heating block with high walls is set to 50 degree centigrade. Mice are placed onto the heating block one at a time. The time from being set onto the heating block to the time the mouse rubs its paws is used as the index for pain sensitivity.

Open field activity

Baseline exploratory behavior in D1 KO mice was tested for motor behaviors with the use of an automated Digiscan apparatus (Accuscan Instruments, Columbus, OH). Mice were handled for three consecutive days for 2 minutes per cage prior to the first day of open field test. Activity is measured by IR beam interruption. Horizontal activity, measured as the total distance traveled by each mouse, was recorded in 1-min intervals over a 10-min period in D1 flx (n = 6), D1 KO (n = 9), D5 flx (n = 9), D5 KO (n = 7), and D1/5 flx (n = 6), D1/5 KO (n = 9) mice, aged 30

– 40 weeks in a novel chamber. This was conducted for three consecutive days. Data collected was averaged across the 1-minute interval by genotype. All experiments were conducted and analyzed blind to the genotypes of the animals. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Motor Activity, D1 KO

Gross motor behavior activity was tested by use of a rotarod apparatus. Mice are placed on a rotating platform that increases in the rate of rotation over a 300 second window. The time from the mice are placed onto the apparatus to the time they fall off is recorded. Mice that do not fall in 300 seconds receive a time of 300 seconds. Mice are given three trials with an approximate inter trial interval of 30min. D1 flx (n = 7), D1 KO (n = 9), D5 flx (n = 7), D5 KO (n = 8), and D1/5 flx (n = 8), D1/5 KO (n = 7), aged 30 – 40 weeks, were run on the rotarod task as explained above. Data collected was averaged and compared by genotype. All experiments were conducted and analyzed blind to the genotypes of the animals.

Delayed Fear Conditioning

Mice were housed in plastic home cages with laboratory bedding (2-4 mice/cage) given *ad libitum* access to food and water with a 12:12 hour light/dark cycle. Contextual fear conditioning and testing were conducted in the same training

environment in the animal facility during the light cycle. All experiments were conducted and analyzed without knowledge of genotypes of the mice.

D1 KO (n=5), D1 flx (n=4), D5 KO (n=9), D5 flx mice (n=7), D1/5 KO (n=7) and D1/5 flx (n=8) mice, between 30 and 40 weeks of age, were transported from the behavioral colony to a holding room adjacent to the behavioral suite containing the fear conditioning chambers. On Day 1 thru Day 3 mice were placed in the holding ram where they sat undisturbed for thirty minutes, after 30 minutes each cage of mice were handled for 2 minutes each. On Day 4 mice were again placed into the holding room for 30 min prior to training and then brought into a room lit with overhead fluorescent lighting and containing four conditioning chambers. The chambers had plexiglass fronts and backs and aluminum sidewalls, and measured 30 x 25 x 21 cm. The chamber floors consisted of 36, 3.2 mm diameter stainless steel rods spaced 7.9mm apart connected via a cable harness to a shock generator. The chambers were cleaned between mice with quatricide and a solution of 1% acetic acid was placed underneath the chambers during the experiment to provide an olfactory cue. All experiments were conducted using FreezeFrame software. Once placed in the chamber the mice freely explored, at 100 seconds a 5000 Hz tone at 50db was given, the tone co-terminated with a single 0.75mA shock with a 2 second duration. Two additional tone-shock pairings were given at 160 and 220 seconds. On Day 2 the mice were returned to an adjacent conditioning room lit with dim red light and placed into

chambers measuring 30 x 25 x 21 cm with a plexiglass front and back and aluminum side walls. However, these chambers contained a white, curved plastic roof and a smooth, white plastic floor. Extensive pilot testing had demonstrated that the replacement of a metal grid with the plastic floor prevented the generalization of the freezing response following single shock conditioning (T. McHugh, unpublished). In addition, the odor in the pan beneath the chamber was switched to 0.25% Benzaldehyde (in 100% EtOH) to further alter the context. Freezing in this chamber was assessed for 5 minutes. On day 3 the mice were returned to the original conditioning chambers (identical to Day 1) for a five-minute test. During all sessions the animal's activity in the chamber was recorded using FreezeFrame software. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Results

Pain Sensitivity

Mice underwent a hotplate test where the time between being placed on a hot plate and when the animal retracts their front paws from the heat is used as the index for pain sensitivity. There is no difference in the time to withdrawal paws in the D1 KO, D5 KO or D1/5 KO mice (Fig. 3-1).

General Anxiety

In order to test general anxiety mice undergo an elevated T-maze test. Mice are placed at the center of an elevated T-maze where two arms have high walls while the other arms do not contain walls. Mice spend a greater amount of time in the high walled alleys vs. the alleys without walls. D5 KO and D1/5 KO mice do exhibit any differences as compared with their flx control counterparts (Fig. 3-2). Mice are also given an open field test to measure anxiety. Mice are given a 10 min. exposure to a novel environment and returned to the same environment for two sequential days for another 10 min. presentation. The time and distance mice spend in the center is a measure of exploration as well as anxiety. There are no differences in the distance mice traverse in the D1 KO, D5 KO or D1/5 KO lines as compared to flx controls (Fig. 3-3 and 4, respectively).

Motor Control

Motor control is examined in mice by a rotarod test. Mice are placed on a rotating arm that increases in rotation rate over a 300 second time period. Mice are each given three tests. D1 KO, D5 KO and D1/5 KO mice do not display significant differences as compared to flx control animals (Fig. 3-5). Mice are also given an open field test where the total distance traveled by each mouse is measured. There is no significant difference in total distance moved between D1 KO, D5 KO and D1/5 KO mice (Fig. 3-4).

Delayed Fear Conditioning

D1, D5 and D1/5 KO show no difference in the acquisition, consolidation or expression of delayed fear conditioning. Mice are given a 300 second three-shock paradigm where each mouse receives a 2 second, 0.75 mA shock initiated at 118, 178 and 238 seconds and each shock co-terminates with a 20 sec tone. Freezing levels during acquisition do not differ between KO animals and flx controls (Fig. 3-6a, 3-7a, 3-8a). 24 hours post-training mice are placed in a distinctly different context from training for 5 minutes, the same tone is presented for 20 seconds and initiated at 100, 160 and 220 seconds, freezing levels between KO animals and flx controls do not differ between genotypes (Fig. 3-6b, 3-7b, 3-8b). 48 hours post training mice are given a 5-minute context test in the

same arena as conditioning. KO mice show no difference in freezing during the context test as compared to controls (Fig. 3-6c, 3-7c, 3-8c).

Discussion

There are no significant deficits as examined by the rotarod test nor are there significant differences in total distance covered in the open field test when the KO lines are compared to flx control animals (Fig. 3-3 and 4). Thus, the behavioral phenotypes (See Chapter 4-6) exhibited by each KO line are not likely due to gross motor deficits. Additionally, KO mice do not display any measurable differences in sensitivity to pain as measured by the pain sensitivity test (Fig. 3-1). General anxiety, as tested by elevated plus maze and by open field, is not significantly different between KO mice and flx controls (Fig. 3-2). Thus, abnormal general anxiety is unlikely to underlie the behavioral phenotypes expressed during contextual fear conditioning and test. Moreover, there are no significant differences in freezing in response to shock during delayed fear conditioning or during the tone and context tests, between genotypes, thus the behavioral phenotypes exhibited by contextual fear conditioning is not likely due to altered sensitivity to shock and amygdalar processing of tone shock associations is intact (Fig. 3-5a, 3-6a, 3-7a). However, given the variability in these measures and the marked decrease in D1R receptor expression in the striatum, it is still a possibility that these animals have deficits that we are not able to capture. Specific linking of the KO to particular learning deficits will only be possible when we elaborate the test battery, varying motor demands and cognitive demands independently, which is a goal for future studies.

Recent reports have shown that mice with genetically lesioned spiny neurons of the striatum, exhibit reduced freezing during a weak delayed fear conditioning paradigm (Kishioka, Fukushima et al. 2009). Results presented in this thesis do not test the same weak training paradigm as in the aforementioned study, where a single 0.3 mA shock resulted in the observed freezing deficits. Thus in future experiments KO mice will be trained and tested on a single 0.3mA shock-training paradigm on delayed fear conditioning. Given a deficit, we may find that the phenotypes observed in the KO lines given a weak training protocol as described in the Kishioka study, could be due to the D1R deletion in the striatum.

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Figure 3-1

Hot Plate Pain Sensitivity Test

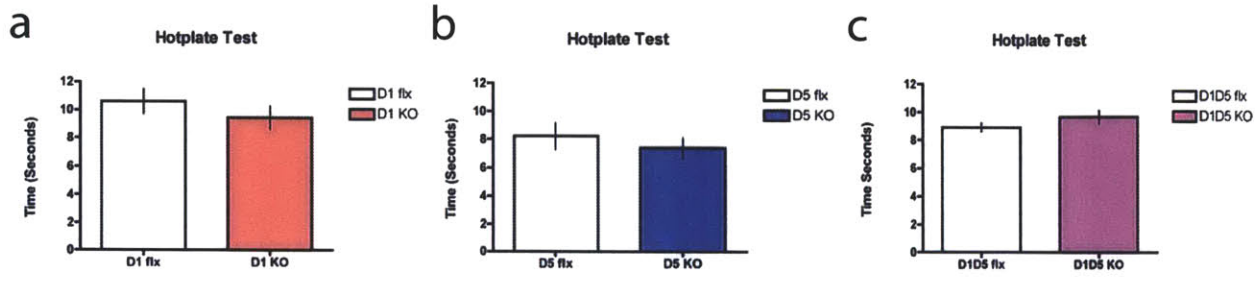


Figure 3-2

Elevated Plus Maze - General Anxiet Test

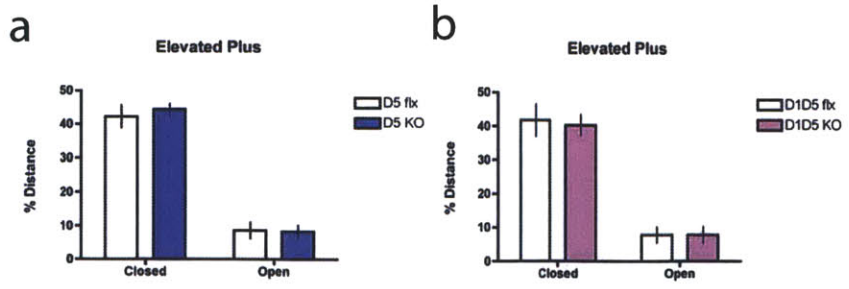


Figure 3-3

Open Field - Center Distance

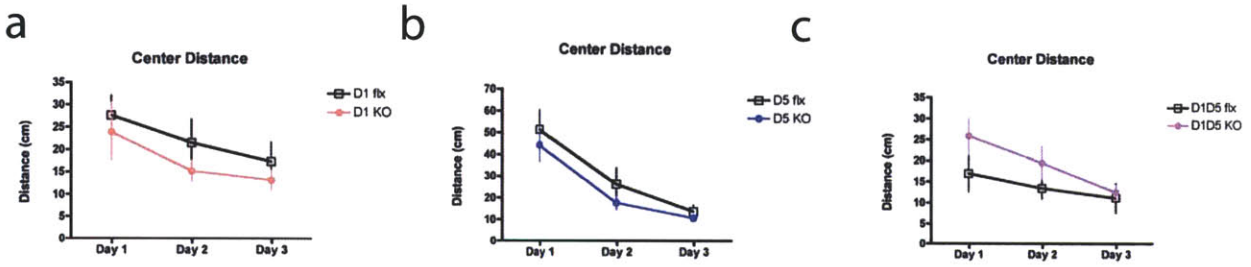


Figure 3-4

Open Field - Total Distance

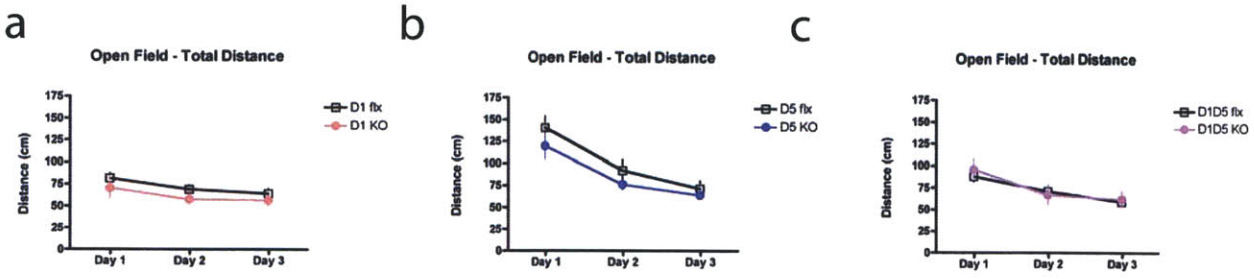
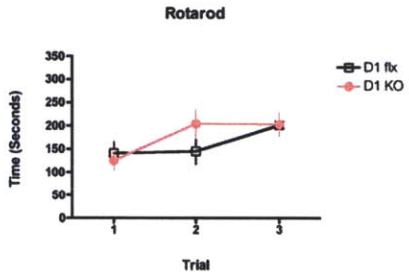


Figure 3-5

Rotarod - General Motor Locomotion Test

a



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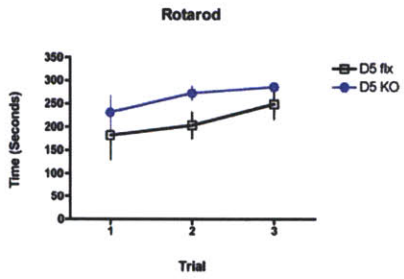


c

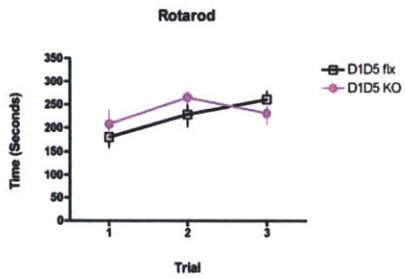


Figure 3-6

Delayed Fear Conditioning - D1 Line

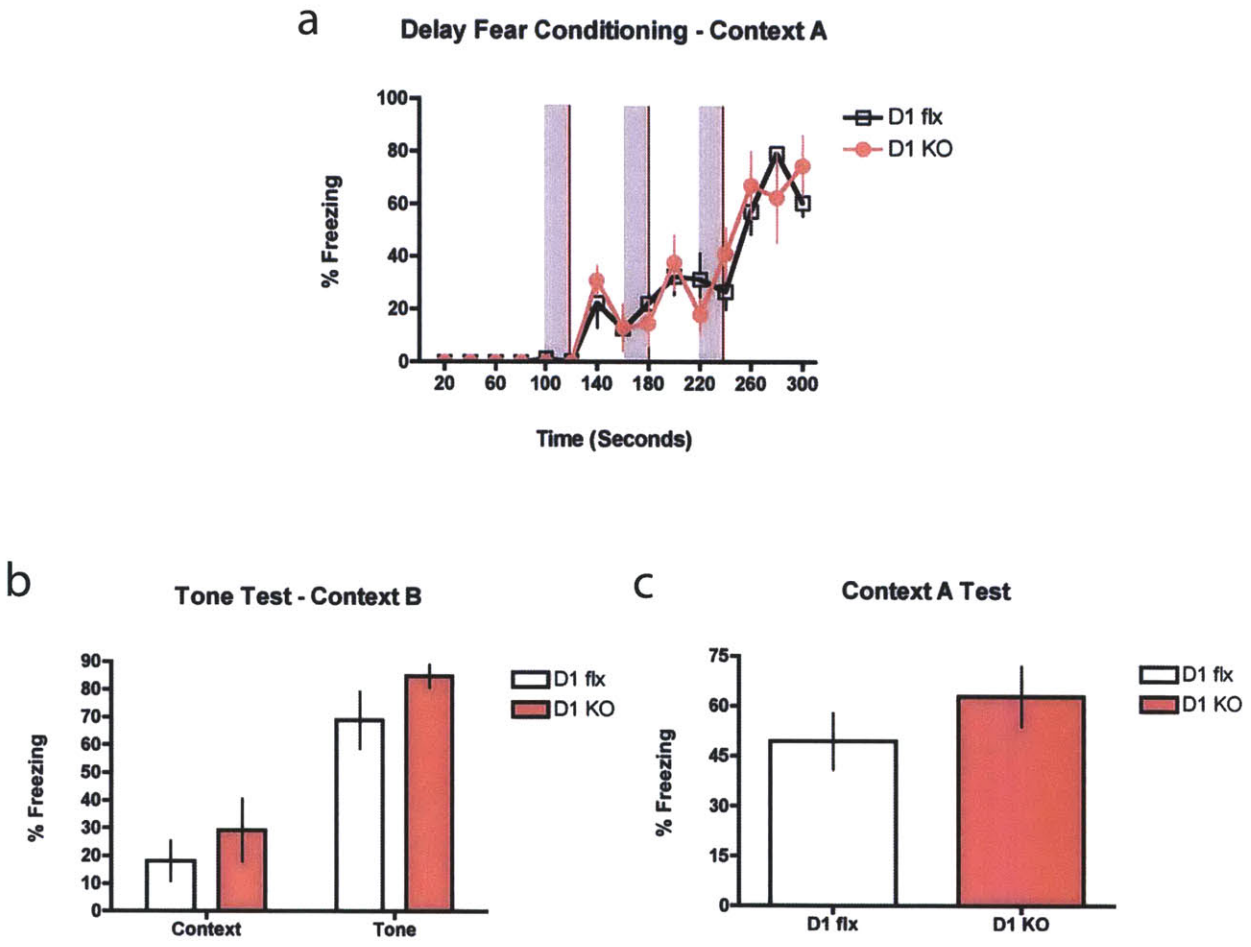


Figure 3-7

Delayed Fear Conditioning - D5 Line

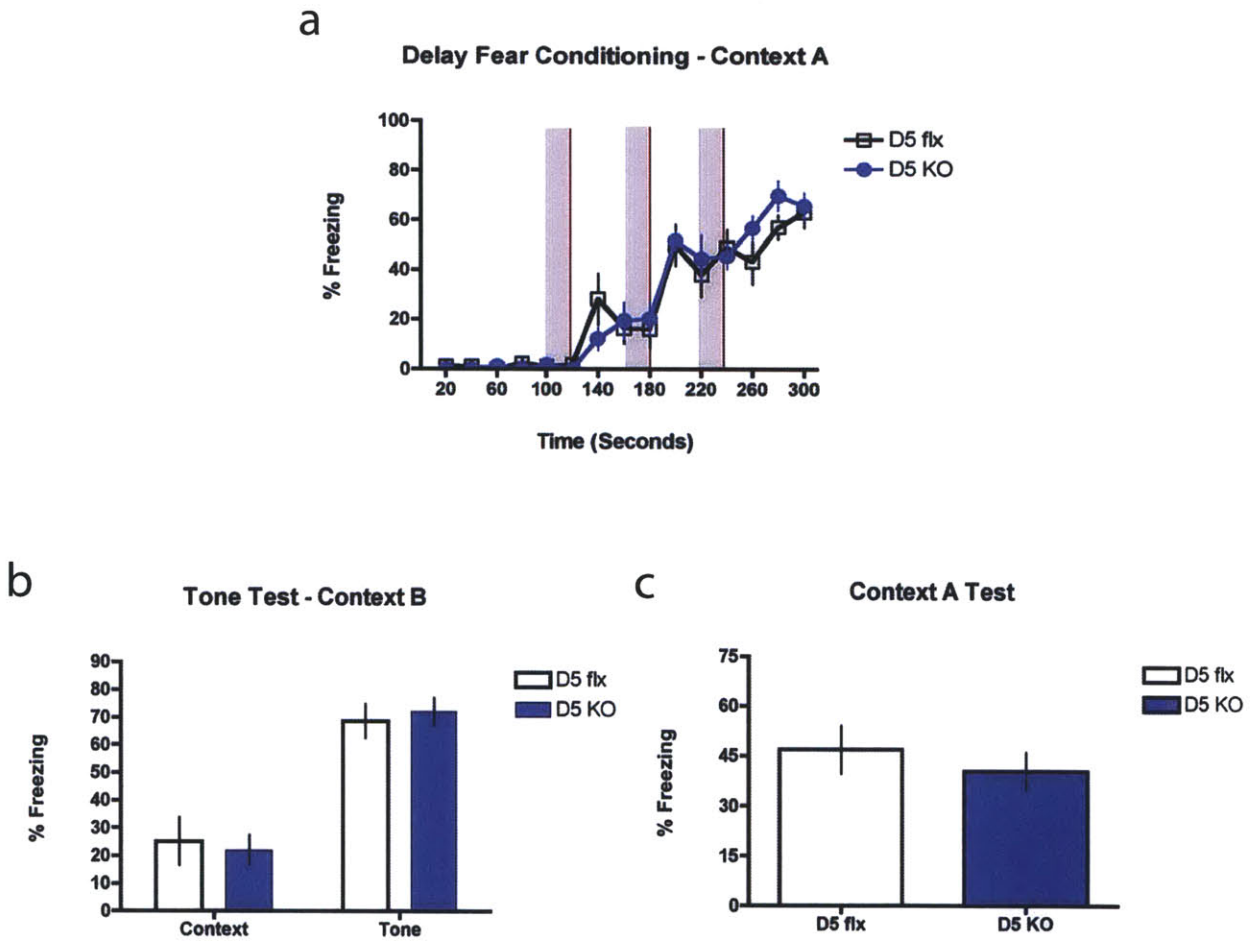
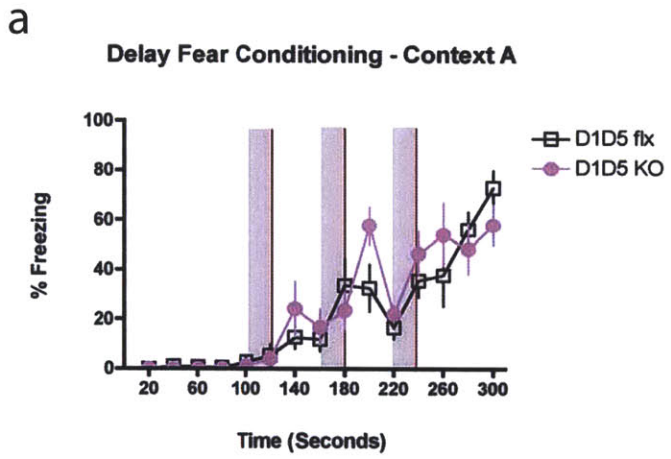
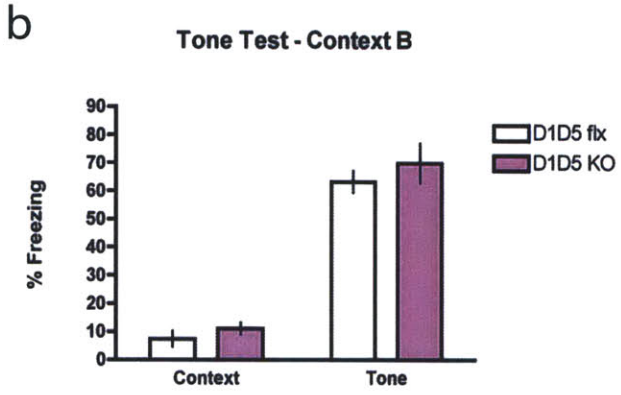


Figure 3-8

Delayed Fear Conditioning - D1/5 Line





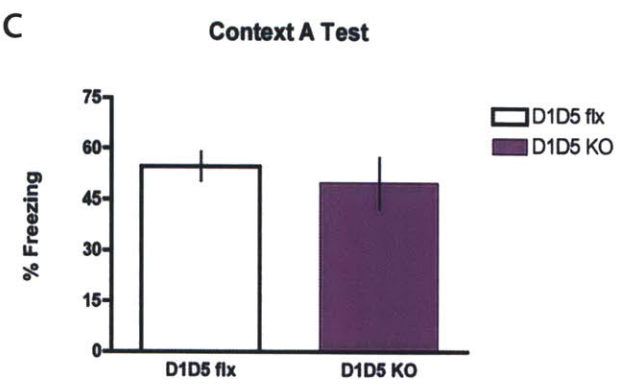


Figure 3-1. Hot Plate Pain Sensitivity Test

(a, b, c) Hot plate sensitivity test for D1 flx (n = 9), D1 KO (n = 8), D5 flx (n = 9), D5 KO (n = 7), and D1/5 flx (n = 10), D1/5 KO (n = 7).

Figure 3-2. Elevated Plus Maze – General Anxiety Test

(a, b) Elevated plus maze, 10 min test duration for D5 flx (n = 7), D5 KO (n = 12) and D1/5 flx (n = 8), D5 KO (n = 7).

Figure 3-3. Open Field – Center Distance

(a, b, c) Open field test, measure of center distance, for D1 flx (n = 6), D1 KO (n = 9), D5 flx (n = 9), D5 KO (n = 7), and D1/5 flx (n = 6), D1/5 KO (n = 9).

Figure 3-4. Open Field – Total Distance

(a, b, c) Open field test, measure of total distance, for D1 flx (n = 6), D1 KO (n = 9), D5 flx (n = 9), D5 KO (n = 7), and D1/5 flx (n = 6), D1/5 KO (n = 9).

Figure 3-5. Rotarod – General Motor Locomotion Test

(a, b, c) Rotarod motor test for D1 flx (n = 7), D1 KO (n = 9), D5 flx (n = 7), D5 KO (n = 8), and D1/5 flx (n = 8), D1/5 KO (n = 7).

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Figure 3-6. Delayed Fear Conditioning – D1 Line

(a) D1 KO mice (n = 5) do not exhibit significant differences in freezing during a three shock delayed fear conditioning protocol as compared to D1 flx mice (n = 4).

(b) D1 KO mice (n = 5) do not exhibit significant differences in freezing during a 24 hour tone fear memory test as compared to D1 flx mice (n = 4).

(c) D1 KO mice (n = 5) do not exhibit significant differences in freezing during a 48 hour context fear memory test as compared to D1 flx mice (n = 4).

Figure 3-7. Delayed Fear Conditioning – D5 Line

(a) D5 KO mice (n = 9) do not exhibit significant differences in freezing during a three shock delayed fear conditioning protocol as compared to D5 flx mice (n = 7).

(b) D5 KO mice (n = 9) do not exhibit significant differences in freezing during a 24 hour tone fear memory test as compared to D5 flx mice (n = 7).

(c) D5 KO mice (n = 9) do not exhibit significant differences in freezing during a 48 hour context fear memory test as compared to D5 flx mice (n = 7).

Figure 3-8. Delayed Fear Conditioning – D1/5 Line

(a) D1/5 KO mice (n = 7) do not exhibit significant differences in freezing during a three shock delayed fear conditioning protocol as compared to D1/5 flx mice (n = 8).

(b) D1/5 KO mice (n = 7) do not exhibit significant differences in freezing during a 24 hour tone fear memory test as compared to D1/5 flx mice (n = 8).

(c) D1/5 KO mice (n = 7) do not exhibit significant differences in freezing during a 48 hour context fear memory test as compared to D1/5 flx mice (n = 8).

Data are presented as mean +/- SEM.

Chapter 4

The Role of D1Rs and D5Rs in Acquisition and Consolidation of Contextual Fear Memories

Abstract

Activation of the D1R family leads to the initiation of gene transcription, a required for memory consolidation. Although the D1R and the D5R share this biochemical pathway, evidence shows distinct activation of non-overlapping pathways between D1Rs and D5Rs. Yet, the independent affect of each receptor subtype on memory consolidation is unknown, as pharmacological agents are unable to agonize or antagonize the D1R independently from the D5R. In order to circumvent this dilemma, mice lacking the D1R and a distinct line of mice lacking the D5R have been created. Neither the D1 KO line nor the D5 KO line exhibit gross differences in motor function, pain sensitivity or anxiety levels. Additionally, each line displays intact delayed fear conditioning, providing evidence that the function of the amygdala is intact. Nevertheless, the D1 and D5 KO's exhibit distinct phenotypes when trained on a either a single or three shock contextual fear conditioning paradigm. When D1 KO mice are trained on a single shock contextual fear paradigm there is a freezing deficit when tested at 24 hours, without freezing deficits observed during training. Unlike the D1 KO mice, the D5 KO's exhibit freezing levels similar to that of the D5 flx control animals during training and during a 24-hour test. However, when trained on a stronger three shock contextual fear paradigm, D1 KO mice exhibit reduced freezing during training, yet they do not display freezing deficits when tested 24 hours later. In contrast, the D5 KO mice exhibits freezing levels during training

that are no different from control mice, however D5 KO mice display enhanced freezing when tested on a LTM test. These data suggest that the D1R and D5R function to create the lower and upper threshold of memory acquisition and consolidation. Thus, the D1R mediates learning when the training episode is less robust, while the D5R constrains learning when the learning episode is very strong. For the first time a distinct hippocampal mediated behavioral phenotype in mice lacking restricted deletion of the D1R and D5R have been demonstrated.

Introduction

The role of the hippocampus in episodic learning, memory and spatial navigation is well established (Morris, Garrud et al. 1982; Kim and Fanselow 1992). Lesions to the hippocampus prior to contextual fear conditioning results in freezing deficits when animal are given a long-term memory test (Kim and Fanselow 1992; Wiltgen, Sanders et al. 2006). In the same vein, hippocampal lesions given prior to training on the Morris water maze results in latency deficits and inhibits memory consolidation of spatial navigation (Morris, Garrud et al. 1982). In addition to structural importance of this brain region, the receptors and biochemical pathways of long-term memory consolidation have been shown to rely on the synthesis of new proteins (Morris, Anderson et al. 1986; Kim, DeCola et al. 1991; Silva, Paylor et al. 1992; Bourtchuladze, Frenguelli et al. 1994; McHugh, Blum et al. 1996; Tsien, Huerta et al. 1996). In particular, the activation of the transcription factor CREB, has been shown to be necessary in the formation of hippocampal LTM of contextual fear (Bourtchuladze, Frenguelli et al. 1994). The activation profile upstream of CREB mediated transcription includes the increased concentration of cAMP, which in turn relies on the stimulation of adenylyl cyclase. The neuromodulator dopamine has been shown to activate the D1R family, which directly leads to the stimulation of adenylyl cyclase and subsequent activation of CREB and gene transcription (Kebabian, Petzold et al.

1972; Smith, Starck et al. 2005). Thus, the role of D1Rs on hippocampal learning and memory has been hypothesized to underlie memory consolidation.

Behavioral studies utilizing the D1R and D5R antagonist, SCH 23390, have provided evidence that these receptors are necessary in the consolidation of hippocampal dependent learning and memory (Inoue, Izumi et al. 2000; Bethus, Tse et al. 2010). When SCH 23390 is given subcutaneously to rats prior to contextual fear conditioning, these animals exhibit freezing deficits when given a context test 24 hours later. However, when SCH 23390 is injected prior to test 24 hours post training there are no freezing deficits (Inoue, Izumi et al. 2000). Thus, pharmacological studies provide evidence that D1/D5Rs are necessary in the acquisition and consolidation of contextual fear. Although D1R and D5R activation initiates the same biochemical pathways leading to CREB mediated transcription, it is not well known as to what role each receptor subtype plays by itself in long-term memory formation and consolidation.

Global deletion of either the D1R or D5R has been utilized in attempts to delineate the function of these receptors in hippocampal memory consolidation (Xu, Moratalla et al. 1994; El-Ghundi, Fletcher et al. 1999; Holmes, Hollon et al. 2001; Granado, Ortiz et al. 2007). D1 -/- mice trained on a contextual fear-conditioning paradigm do not display freezing deficits during training or during a 24 hour-long term memory test (El-Ghundi, Fletcher et al. 1999). However, D1 -/-

exhibit deficits in spatial navigation when trained and tested on the Morris water maze (Granado, Ortiz et al. 2007). In addition, D5 -/- mice do not reveal deficits in delayed fear conditioning or in spatial navigation (Holmes, Hollon et al. 2001). These results show that the D1R is necessary in spatial navigation but not contextual fear while the D5R is not necessary for either.

Three general interpretations can be provided; first, it may be that the combined deletion of both the D1R and D5R is necessary in order to produce deficits that parallel the pharmacological findings in contextual fear conditioning utilizing SCH 23390. Second, given the global deletion of these receptors, homeostatic affects likely ameliorate deficits shown by the antagonist studies. Third, it may be the case that these receptors are not necessary and the pharmacological data does not accurately depict the necessity of the D1R and D5R in memory consolidation. In order circumnavigate these differences I have characterized mice with regional deletions of the D1R or D5R. I conclude that the D1R is necessary in the consolidation contextual fear conditioning when the training paradigm consists of a single shock versus a three shock contextual fear paradigm. In addition, the D5R functions to reduce the acquisition or consolidation of contextual fear when the given a three shock, but not a single shock, training paradigm.

Methods and Materials

One-trial contextual fear conditioning, LTM

Mice were housed in plastic home cages with laboratory bedding (2-4 mice/cage) given *ad libitum* access to food and water with a 12:12 hour light/dark cycle. Contextual fear conditioning and testing were conducted in the same training environment in the animal facility during the light cycle. All experiments were conducted and analyzed without knowledge of genotypes of the mice. D1 KO (n = 11), D1 flx (n = 11), D5 KO (n = 5) and D5 flx (n=8) mice, between 30 and 30 weeks of age, were transported from the behavioral colony to a holding room adjacent to the behavioral suite containing the fear conditioning chambers. On Day 1 thru Day 3 mice were placed in the holding ram where they sat undisturbed for thirty minutes, after 30 minutes each cage of mice were handled for 2 minutes each. On Day 4 mice were again placed into the holding room for 30 min prior to training and then brought into a room lit with overhead fluorescent lighting and containing four conditioning chambers. The chambers had plexiglass fronts and backs and aluminum sidewalls, and measured 30 x 25 x 21 cm. The chamber floors consisted of 36, 3.2 mm diameter stainless steel rods spaced 7.9mm apart connected via a cable harness to a shock generator. The chambers were cleaned between mice with quatricide and a solution of 1% acetic acid was placed underneath the chambers during the experiment to provide an olfactory

cue. All experiments were conducted using FreezeFrame software. Once placed in the chamber the mice freely explored for 238 seconds, and then received a single, unsignaled 1 mA footshock (2 sec in duration). Following the shock the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. On Day 5 the mice were returned to same conditioning room and chambers as on day 4. The odor in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Three-trial contextual fear conditioning, STM

The same habituation, handling, conditioning chambers and odor cues were utilized as in the one-trial conditioning paradigm. 9 D5 KO male mice and 7 flx D5 littermate controls between 30 and 40 weeks of age were used in this experiment. Once placed in the chamber the mice freely explored for 118 seconds, and then received a single, unsignaled 0.75 mA footshock (2 sec in duration). Two additional shocks of the same amplitude and duration were given at 178 and 238 seconds. Following the shocks the mice remained in the

chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. One hour later the mice were returned to same conditioning room and chambers. The odor in the pan underneath the chamber was and lighting was the same as during training. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Three-trial contextual fear conditioning, LTM

The same habituation, handling, conditioning chambers and odor cues were utilized as in the one-trial conditioning paradigm. D1 KO (n = 8), D1 flx (n = 9), D5 KO (n = 15) and D5 flx (n = 15) mice, between 30 and 40 weeks of age were used in this experiment. Once placed in the chamber the mice freely explored for 118 seconds, and then received a single, unsignaled 0.75 mA footshock (2 sec in duration). Two additional shocks of the same amplitude and duration were given at 178 and 238 seconds. Following the shocks the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. On Day 5 the mice were returned to same conditioning room and chambers as on day 4. The odor

in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Results

D1Rs are Required for the Acquisition and Consolidation of Single Shock Contextual Fear Conditioning

Contextual fear conditioning is a hippocampal dependent learning paradigm that can be utilized to study the acquisition, consolidation and expression of learning and memory. Therefore I trained mice on single 1 mA shock contextual fear-training paradigm. D1 KO mice trained on this paradigm exhibit similar freezing during training as compared to littermate controls, however when given a long term memory test 24 hours later, D1 KO mice exhibit significantly reduced freezing (Fig. 4-1a, b; $p < 0.05$, compare to flx, Student's t-test; c; two-way ANOVA (genotype x time) $F(1,9) = 1.1$, $p = 0.40$; genotype $F(1,9) = 6.2$, $p < 0.05$; time $F(1,9) = 3.4$, $p < 0.0001$) . Previous research has shown that stronger training in contextual fear can overcome hippocampal lesions and in order to test if stronger training would ameliorate freezing deficits mice were trained on a three shock, 0.75 mA, contextual fear-training paradigm (Wiltgen, Sanders et al. 2006). D1 KO mice exhibit significantly reduced freezing during training, however during the 24 Hr test, D1 KO mice do not exhibit freezing deficits as compared to flx D1 controls (Fig. 4-2a; two-way ANOVA (genotype x time interaction) $F(1,9) = 10.52$, $p < 0.0001$; b). These data suggest that the D1R is necessary for memory consolidation for the single shock but not for the stronger three shock training

paradigm, showing that learning deficits can be overcome given appropriate training.

D5Rs Reduce CS-US Associations In Contextual Fear Conditioning

Both the D1R and D5R activate adenylyl cyclase leading to CREB mediated transcription. CREB is known to be required for memory consolidation and in order to test the role of D5Rs in the acquisition, consolidation and expression of learning and memory mice were trained on the same single and three shock contextual fear paradigm as D1 KO experiment. D5 KO mice exhibit freezing levels that are not significantly different during training or during a 24 Hr LTM test (Fig. 4-3). However, D5 KO mice freeze significantly more during a 24 Hr test on the three shock training paradigm (Fig. 4-4b; $p < 0.05$, compare to flx, Student's t test, c; two-way ANOVA (genotype x time) $F(1,9) = 1.2$, $p = 0.33$; genotype $F(1,9) = 6.1$, $p < 0.05$; time $F(1,9) = 9.2$, $p < 0.0001$)). The enhanced freezing is unlikely due to increased sensitivity to shocks as D5 KO animals display freezing levels that are not significantly different from flx D5 animals during training (Fig. 4-4a). Furthermore, when given a 1 Hr STM test after the three shock training paradigm, D5 KO animals do not differ in freezing as compared to controls (Fig. 4-5). These date show the D5 KO animals exhibit enhanced freezing during a LTM test, which suggests enhanced memory acquisition and/or consolidation.

Thus, hippocampal D5Rs are important in constraining the strength of CS-US associations.

Discussion

D1 KO animals exhibit freezing deficits during a 24 Hr, single shock training paradigm, yet they do not exhibit freezing differences during a 24 Hr test on a three shock-training paradigm (Fig. 4-1 and 2, respectively). Additionally, D5 KO animals do not exhibit differences in freezing during a 24 Hr test on a single shock training paradigm, but do exhibit enhanced freezing on a three shock training paradigm LTM test (Fig. 4-3 and 4, respectively). I conclude that the D1R is required for the acquisition or consolidation of a weak-learning experience, however these deficits can be ameleriorated given stronger training, while the D5R is required to reduce the impact of strong learning episodes. Thus, the D1Rs and D5Rs function to set a lower and upper bounds to memory acquisition and consolidation.

The importance of D1R and D5R mediating weak and strong learning was not directly tested in these experiments. However, these findings may be important in cue mediated drug relapse. Environmental cues associate to the emotional and cognitive states, in humans, during drug use. Individuals with drug addiction that no longer use are susceptible to cue induced drug relapse as cues can induce drug craving seeking. Given the important of cues and contextual association in emotional and cognitive states, studying how the hippocampus may mediate the ability of cue's to induce drug craving and seeking is an

important endeavor. The development of cued associations to the emotional and cognitive state during drug use may be mediated by D1R and D5R activation as DA release is increased during drug use. The data above provides evidence that shows that D1Rs and D5Rs play a significant role in developing CS-US association. Thus, D1R activation may be important in creating CS-US associations that are weak while D5R activation may be important in preventing CS-US associations in becoming too strong. In the context of drug addiction, the D1/5R upper and lower bound CS-US function, may be significantly altered such that associations between cues and drug use may become too strong, a result of reduced D5R function. Similarly, when no longer a drug user, the individual may find it difficult to form new cued associations, due to altered D1R function as a drug user, as D1Rs function to mediated weaker CS-US associations.

These data provide the first behavioral phenotype allocated to either the D1R or the D5R in a delineated manner. Previous pharmacological studies utilizing SCH 23390 and studies using D1 -/- and D5 -/- have not been able to accurately and precisely lesion the D1R or D5R and thus have not been able to test the specific aspects of these receptors in hippocampal dependent contextual learning and memory (Xu, Moratalla et al. 1994; El-Ghundi, Fletcher et al. 1999; Inoue, Izumi et al. 2000; Granado, Ortiz et al. 2007). SCH 23390 studies fail to differentiate D1R function from D5R function in that SCH 23390 is an antagonist to both D1Rs and D5Rs. Global KO studies are not able to spatially restrict the

deletion of either the D1R or D5R, thus behavioral phenotypes in these mice are not able to attribute observed phenotypes to hippocampal processing. The D1R KO and D5R KO offer the most regionally restricted deletion of the D1R and D5R and offers the ability to differentiate the function of D1Rs and D5Rs in hippocampal dependent learning and memory.

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Figure 4-1

Single Shock Contextual Fear Conditioning - D1 Line, Long Term Memory Test

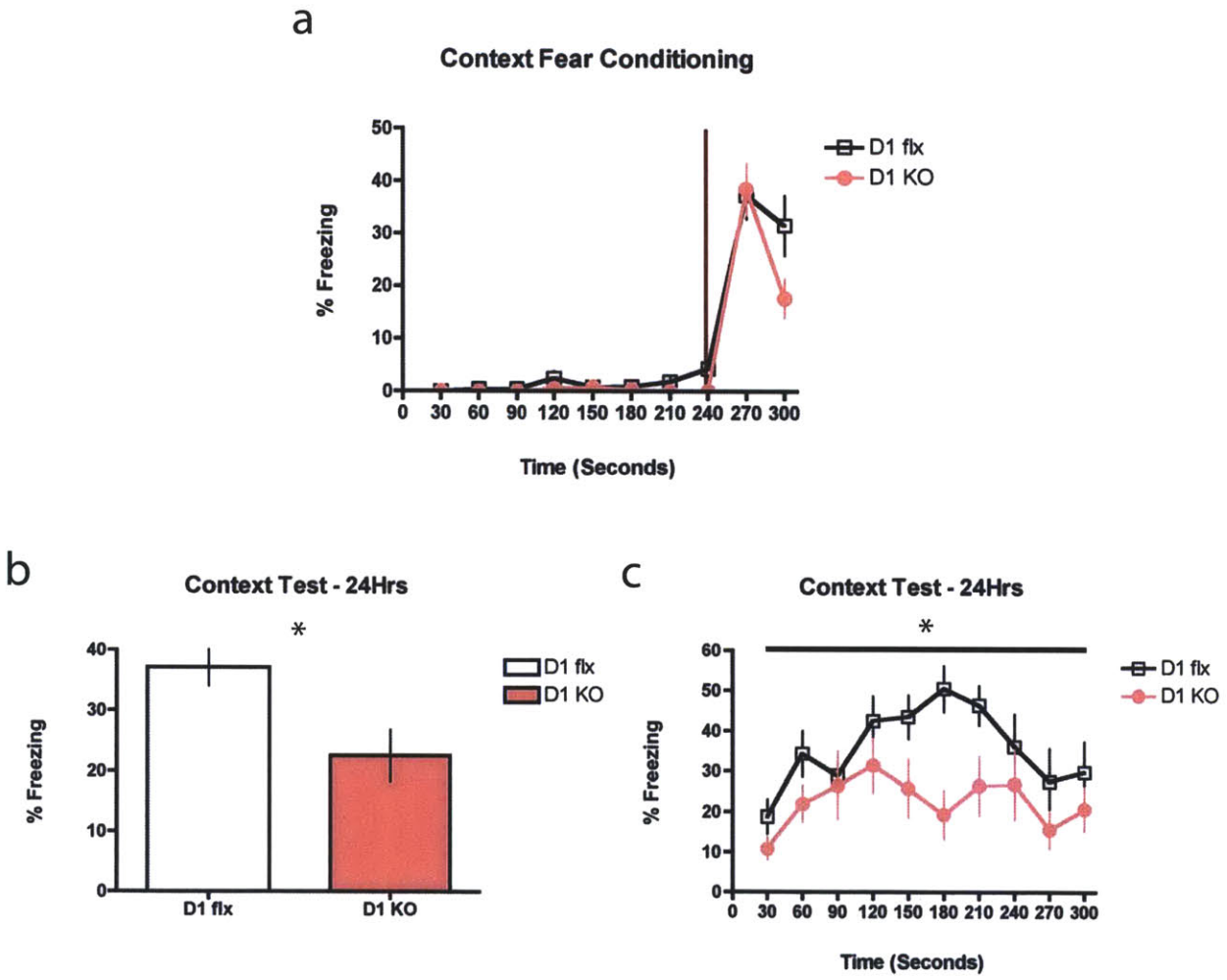


Figure 4-2

Three Shock Contextual Fear Conditioning - D1 Line, Long Term Memory Test

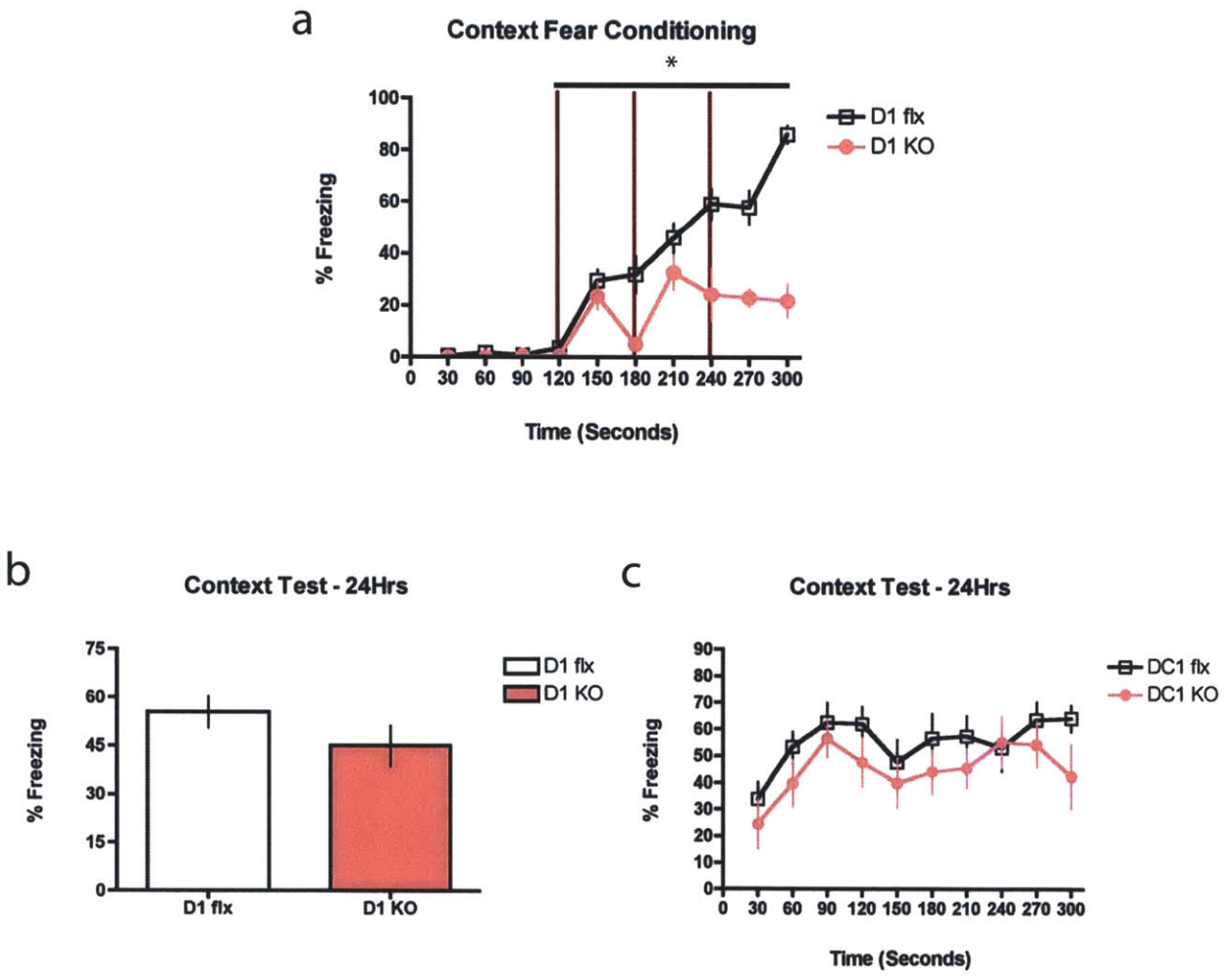
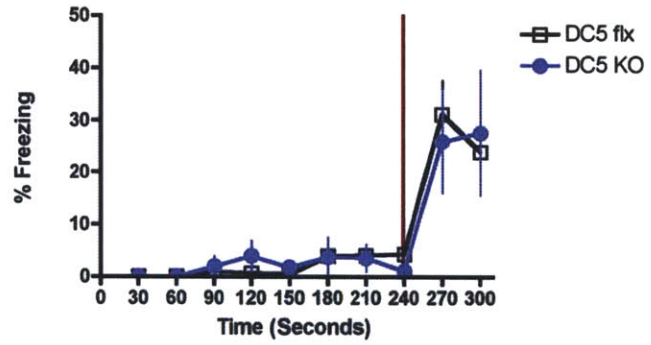


Figure 4-3

Single Shock Contextual Fear Conditioning - D5 Line, Long Term Memory Test

a



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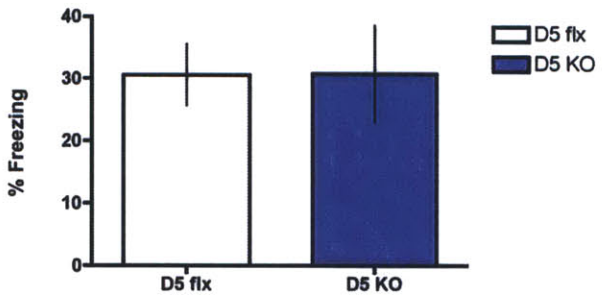

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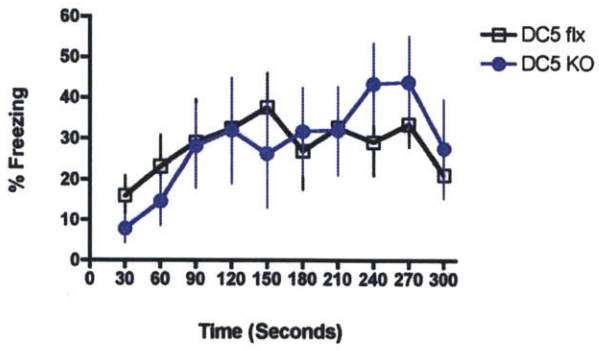

Figure 4-4

Three Shock Contextual Fear Conditioning - D5 Line, Long Term Memory Test

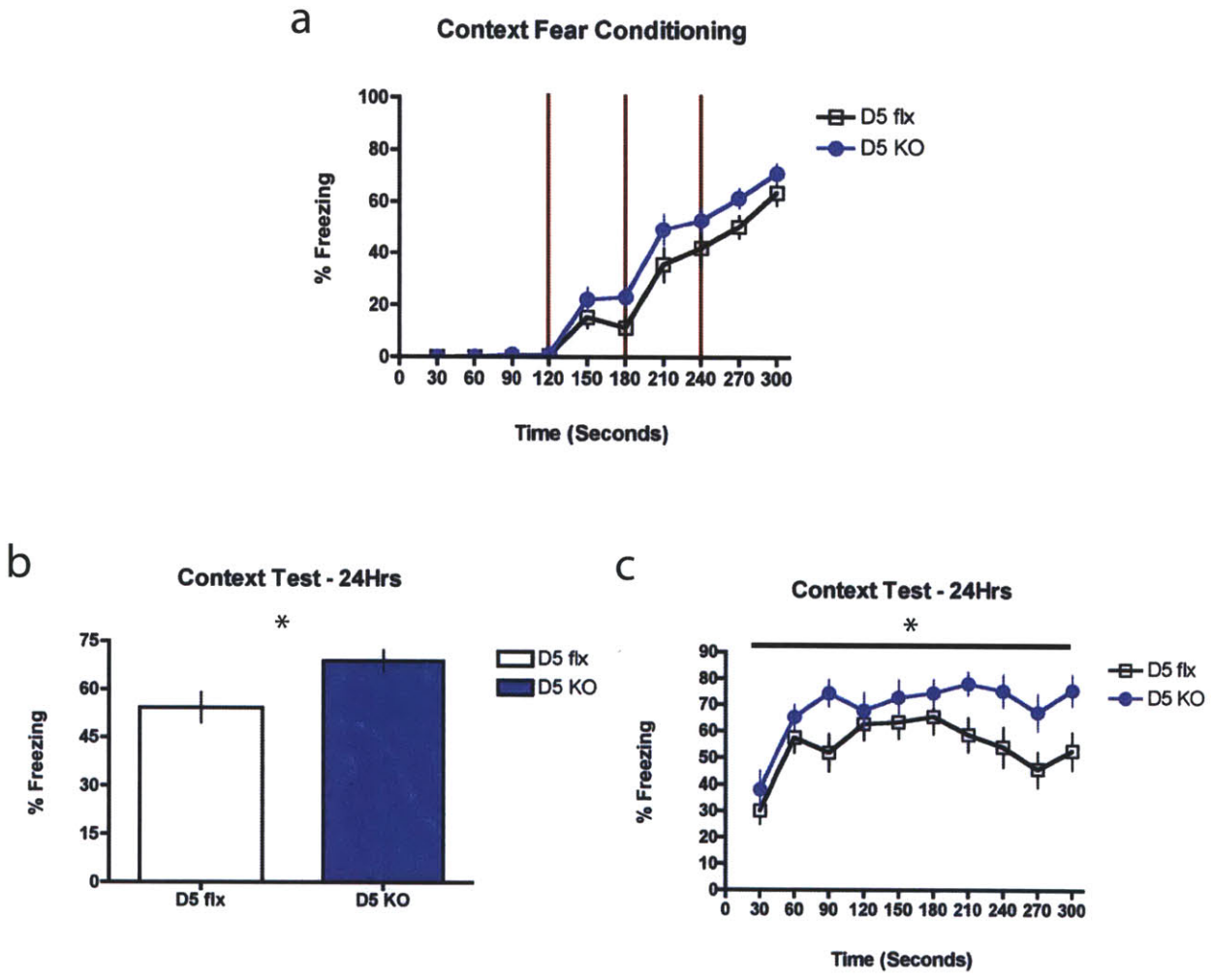

Figure 4-5

Three Shock Contextual Fear Conditioning - D5 Line, Short Term Memory Test

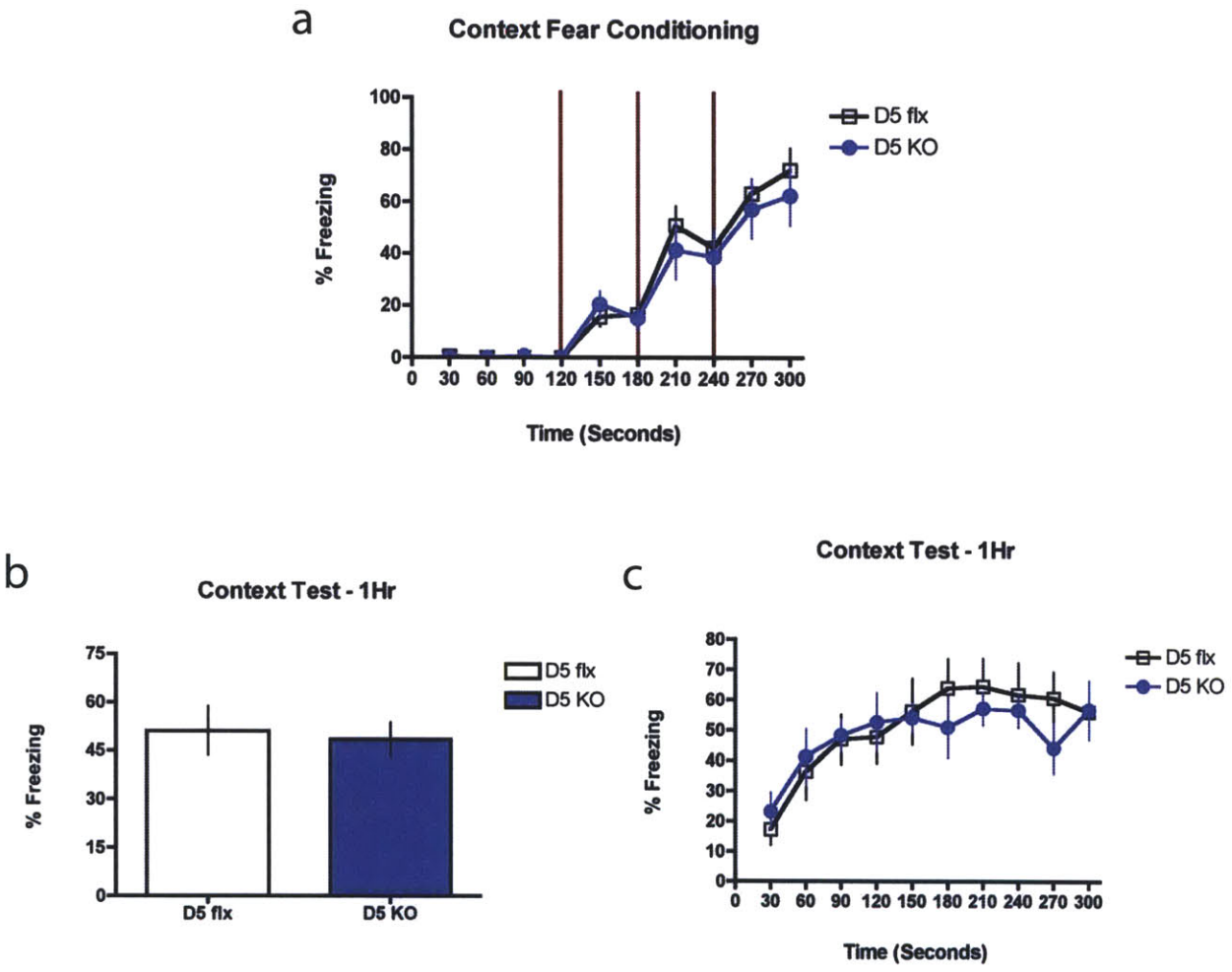

Figure 4-1. Single Shock Contextual Fear Conditioning – D1 Line, Long Term Memory Test

(a) Context Fear Conditioning. Single shock, 1 mA, context fear paradigm. D1 KO mice (n = 11) and D1 flx (n = 11).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1 KO (n = 11) freeze significantly less ($p < 0.05$) during test as compared to D1 flx (n = 11).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 4-2. Three Shock Contextual Fear Conditioning – D1 Line, Long Term Memory Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D1 KO mice (n = 8) exhibit significantly less freezing ($p < 0.0001$) during training as compared to D1 flx (n = 9)

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1 KO (n = 8) and D1 flx (n = 9).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 4-3. Single Shock Contextual Fear Conditioning – D5 Line, Long Term Memory Test

(a) Context Fear Conditioning. Single shock, 1 mA, context fear paradigm. D5 KO (n = 5) and D5 flx (n = 8).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. . D5 KO (n = 5) and D5 flx (n = 8).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 4-4. Three Shock Contextual Fear Conditioning – D5 Line, Long Term Memory Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D5 KO (n = 15) and D5 flx (n = 15).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D5 KO mice (n = 15) freeze significantly more ($p < 0.05$) during test as compared to D1 flx (n = 15).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 4-5. Three Shock Contextual Fear Conditioning – D5 Line, Short Term Memory Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D5 KO (n = 7) and D5 flx (n = 9).

(b) 1 Hr Context Test. 5 min. test 1 hour post training. D5 KO (n = 7) and D5 flx (n = 9).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Data are presented as mean +/- SEM.

Chapter 5

The Comparison of D1/5 KO Mice in Contextual Fear Acquisition and Consolidation to SCH 23390 Pharmacological Studies

Abstract

SCH 23390 is a pharmacological antagonist to both the D1R and D5R. In order to test the accuracy of SCH 23390 in blocking D1/5Rs we have characterized mice lacking D1/5Rs. We show that in contrast to SCH 23390 studies; D1/5 KO animals do not exhibit deficits in the acquisition, consolidation or expression of STM or LTM of contextual fear learning and memory. Deficits are not observed whether conditioning is on a single shock or three shock training paradigm. However, when tested on an extinction protocol D1/5 KO animals exhibit altered fear extinction when trained on a single shock training protocol, yet there are no differences when fear extinction follows a three shock training protocol. Additionally, when animals are trained on a three shock paradigm and subsequently tested seven days later, D1/5 KO animals do not differ in freezing levels when compared to control littermates, providing evidence that D1/5Rs are not necessary for the maintenance of fear memory, which has previously been suggested. These data are in stark contrast to the pharmacological data utilizing SCH 23390 in behavioral studies, we show that D1/5Rs are not necessary for the acquisition, consolidation and expression of fear memory whether the training is of single or mutli shock training protocol. I show, for the first time, that D1/5Rs are important in modulating extinction of a weak fear conditioning and suggest that the reduced freezing observed during extinction is due to instability of the memory trace.

Introduction

SCH 23390 is a pharmacological agent that has been used to understand the roles of D1Rs and D5Rs in hippocampal dependent learning and memory (Inoue, Izumi et al. 2000; Lemon and Manahan-Vaughan 2006; O'Carroll, Martin et al. 2006; Moncada and Viola 2007; Rossato, Bevilaqua et al. 2009). SCH 23390 is a potent D1/5R antagonist that has been shown to block the consolidation of contextual fear memories, but not the expression of contextual fear (Inoue, Izumi et al. 2000). D1/5Rs antagonist studies have also shown that these receptors are necessary for the maintenance of hippocampal dependent inhibitory avoidance memory (Rossato, Bevilaqua et al. 2009). In addition, SCH 23390 studies have shown that D1/5Rs are necessary for detecting novelty and are activated by novelty exposure, which can modulate the induction of synaptic plasticity (Li, Cullen et al. 2003; Lemon and Manahan-Vaughan 2006; Moncada and Viola 2007). Moreover, D1/5Rs have been shown to be necessary and sufficient for synaptic plasticity as well as for inhibiting depotentiation of potentiated synapses (Xu, Anwyl et al. 1998). These data offer the view that D1/5R activation is necessary for memory consolidation, necessary for maintaining the persistence of long-term memory storage and necessary for novelty detection. These behavioral studies also point to the necessity of D1/5Rs in the induction and maintenance of synaptic potentiation.

Although the D1/5R antagonist SCH 23390 has been used in pointing to the necessity of D1/5R in hippocampal dependent learning and memory, there is lack of specificity of SCH 23390 antagonism to other receptors. SCH 23390 has also been shown to bind to serotonergic receptors, primarily 5-HT_{1c} and 5-HT_2, and exhibits weak affinity to 5-HT_{1B}, 5-HT_{1A}, as well as to the alpha 1-adrenergic receptors (Hicks, Schoemaker et al. 1984; Bischoff, Heinrich et al. 1986; Woodward, Panicker et al. 1992). Furthermore, SCH 23390 has been shown to enhance the serotonergic system by acting as a serotonin reuptake inhibitor, and serotonin reuptake inhibitors have been shown to inhibit the induction of LTP (Shakesby, Anwyl et al. 2002; Kojima, Matsumoto et al. 2003; Zarrindast, Honardar et al. 2011). The 5-HT_2R has been shown to affect hippocampal LTP in area CA1 of the hippocampus, such that its inactivation results in blockade of CA1 LTP in rats that have been previously stressed (Ryan, Anwyl et al. 2008). This finding is particularly important considering that acute stress rodents experience in contextual fear conditioning. Additionally, SCH 23390 has been shown to act as an agonist to 5-HT_{1c}Rs in xenopus oocytes (Briggs, Pollock et al. 1991). Moreover, 5-HT_{1c}Rs are highly expressed in all hippocampal subregions, suggesting that SCH 23390 affects occur throughout the hippocampus (Hoffman and Mezey 1989). Thus, the affects of the benchmark antagonist, SCH 23390, are not necessarily due to the inhibition of D1Rs and D5Rs. 5-HT_2Rs and 5-HT_{1c}Rs are significantly affected by SCH 23390, a compound used to study the role of D1/5Rs. Given the affects of SCH 23390 on D1Rs, D5Rs, 5-HT_2Rs and 5-

HT_{1c}Rs I conclude that the role of D1/5Rs on hippocampal dependent learning, memory and plasticity is still unknown and at best ambiguous.

Our results utilizing region specific D1/5 KO mice are in stark contrast to the SCH 23390 literature on the role of D1/5Rs in hippocampal dependent learning and memory. We show that D1/5Rs are not necessary for the acquisition, consolidation and expression of contextual fear conditioning, supporting the evidence that SCH 23390 is not only non-selective to D1/5Rs but also provides deleterious results to the field.

Methods and Materials

One-trial contextual fear conditioning (0.5 mA) – Long Term Memory Test and Extinction

Mice were housed in plastic home cages with laboratory bedding (2-4 mice/cage) given *ad libitum* access to food and water with a 12:12 hour light/dark cycle. Contextual fear conditioning and testing were conducted in the same training environment in the animal facility during the light cycle. All experiments were conducted and analyzed without knowledge of genotypes of the mice. 11 D1/5 KO and 10 D/15 flx mice between 30 and 40 weeks of age were transported from the behavioral colony to a holding room adjacent to the behavioral suite containing the fear conditioning chambers. On Day 1 thru Day 3 mice were placed in the holding room where they sat undisturbed for 30 minutes and subsequently handled for 2 minutes each. On Day 4 mice were again placed into the holding room for 30 min prior to training and then brought into a room lit with overhead fluorescent lighting and containing four conditioning chambers. The chambers had plexiglass fronts and backs and aluminum sidewalls, and measured 30 x 25 x 21 cm. The chamber floors consisted of 36, 3.2 mm diameter stainless steel rods spaced 7.9mm apart connected via a cable harness to a shock generator. The chambers were cleaned between mice with quatricide and a solution of 1% acetic acid was placed underneath the chambers during the

experiment to provide an olfactory cue. All experiments were conducted using FreezeFrame software. Once placed in the chamber the mice freely explored for 178 seconds, and then received a single, unsignaled 0.5 mA footshock (2 sec in duration). Following the shock the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. On Day 5 the mice were returned to same conditioning room and chambers as on day 4. The odor in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 15 minutes. The first 3 minutes of freezing was used as an index of memory recall, while the full 15 minutes of freezing was used as a measuring of fear extinction. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

One-trial contextual fear conditioning (1 mA) – Long Term Memory Test

Mice were housed in plastic home cages with laboratory bedding (2-4 mice/cage) given *ad libitum* access to food and water with a 12:12 hour light/dark cycle. Contextual fear conditioning and testing were conducted in the same training environment in the animal facility during the light cycle. All experiments were

conducted and analyzed without knowledge of genotypes of the mice. 6 D1/5 KO and 8 D/15 flx mice between 30 and 40 weeks of age were transported from the behavioral colony to a holding room adjacent to the behavioral suite containing the fear conditioning chambers. On Day 1 thru Day 3 mice were placed in the holding room where they sat undisturbed for 30 minutes and subsequently handled for 2 minutes each. On Day 4 mice were again placed into the holding room for 30 min prior to training and then brought into a room lit with overhead fluorescent lighting and containing four conditioning chambers. The chambers had plexiglass fronts and backs and aluminum sidewalls, and measured 30 x 25 x 21 cm. The chamber floors consisted of 36, 3.2 mm diameter stainless steel rods spaced 7.9mm apart connected via a cable harness to a shock generator. The chambers were cleaned between mice with quatricide and a solution of 1% acetic acid was placed underneath the chambers during the experiment to provide an olfactory cue. All experiments were conducted using FreezeFrame software. Once placed in the chamber the mice freely explored for 238 seconds, and then received a single, unsignaled 1 mA footshock (2 sec in duration). Following the shock the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. On Day 5 the mice were returned to same conditioning room and chambers as on day 4. The odor in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the

video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Three-trial contextual fear conditioning – Short Term Memory Test

The same habituation, handling, conditioning chambers and odor cues were utilized as in the one-trial conditioning paradigm. 9 D1/5 KO and 9 D/15 flx mice between 30 and 40 weeks of age were transported from the behavioral colony to a holding room adjacent to the behavioral suite containing the fear conditioning chambers. On Day 1 thru Day 3 mice were placed in the holding room where they sat undisturbed for 30 minutes and subsequently handled for 2 minutes each. On Day 4 mice were again placed into the holding room for 30 min prior to training and then brought into a room lit with overhead fluorescent lighting and containing four conditioning chambers. The chambers had plexiglass fronts and backs and aluminum sidewalls, and measured 30 x 25 x 21 cm. The chamber floors consisted of 36, 3.2 mm diameter stainless steel rods spaced 7.9mm apart connected via a cable harness to a shock generator. The chambers were cleaned between mice with quatricide and a solution of 1% acetic acid was placed underneath the chambers during the experiment to provide an olfactory cue. All experiments were conducted using FreezeFrame software. Once placed

in the chamber the mice freely explored for 118 seconds, and then received a single, unsignaled 0.75 mA footshock (2 sec in duration). Two additional shocks of the same amplitude and duration were given at 178 and 238 seconds. Following the shocks the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. At the conclusion of the session they were returned to their home cages and remained in the holding room for 1 Hr. 1 Hr post training mice were returned to same conditioning room and chambers as during training. The odor in the pan underneath the chamber was and lighting was the same as during training. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007).

Three-trial contextual fear conditioning – Long Term Memory Test and Extinction

The same habituation, handling, conditioning chambers and odor cues were utilized as in the one-trial conditioning paradigm. 11 D1 KO male mice and 9 flx D1 littermate controls between 30 and 40 weeks of age were used in this experiment. Once placed in the chamber the mice freely explored for 118

seconds, and then received a single, unsignaled 0.75 mA footshock (2 sec in duration). Two additional shocks of the same amplitude and duration were given at 178 and 238 seconds. Following the shocks the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room. On Day 5 the mice were returned to same conditioning room and chambers as on day 4. The odor in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 15 minutes. The first 3 minutes of freezing was used as an index of memory recall, while the full 15 minutes of freezing was used as a measuring of fear extinction. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007).

Long Term Memory Maintenance Training and Test

The same habituation, handling, conditioning chambers and odor cues were utilized as in the one-trial conditioning paradigm. 7 D1 KO male mice and 4 flx D1 littermate controls between 30 and 40 weeks of age were used in this experiment. Once placed in the chamber the mice freely explored for 118 seconds, and then received a single, unsignaled 0.75 mA footshock (2 sec in

duration). Two additional shocks of the same amplitude and duration were given at 178 and 238 seconds. Following the shocks the mice remained in the chamber for one minute. At the conclusion of the session they were returned to their home cages and transported back to the holding room where animals remained for 7 days. On Day 11 the mice were returned to same conditioning room and chambers as on day 4. The odor in the pan underneath the chamber was and lighting was the same as on day 4. Freezing in this chamber was assessed for 5 minutes. Freezing behavior was assessed from the video image of the mouse using FreezeView software, with a minimum bout time of 1.25 seconds. Freezing values were then averaged over mice of a particular genotype for each session. Adapted from Thomas J. McHugh (McHugh, Jones et al. 2007)

Results

Acquisition, Consolidation and Expression of Conditioned Fear are Intact in D1/5 KO Mice

Contextual fear conditioning experiments in rats with subcutaneous SCH 23390 injections exhibit deficits in the acquisition and consolidation of contextual fear (Inoue, Izumi et al. 2000). In order to test the role of D1/5Rs in contextual fear conditioning, mice were trained on several contextual fear conditioning paradigms, that ranged from weak to strong training, in order to determine the intensity of the contextual fear paradigm that may lead to deficits in mice that replicate the SCH 23390 study. The first paradigm consisted of a single 0.5 mA shock during a 3 minute training protocol, the second paradigm consisted of a single 1 mA shock during a 5 minute training protocol and the third paradigm consisted of three 0.75 mA shocks during a 5 minute training protocol. D1/5 KO mice trained on the first paradigm (a single 0.5 mA shock) exhibited similar freezing during training as compared to littermate controls and when given a LTM test 24 Hr later, D1/5 KO mice exhibit similar freezing levels during a three minute contextual fear memory test (Fig. 5-1). The same mice remained in the test environment for an additional 12 minutes as an extinction session. Although there were no significant differences in freezing during the LTM test (Fig. 5-1b,c), D1/5 KO mice exhibited significantly reduce freezing during the 15 minute

memory extinction session (Fig. 5-2b; $p < 0.005$, compare to flx, Student's t test, c; two-way ANOVA (genotype x time) $F(1,29) = 1.2$, $p = 0.27$; genotype $F(1,29) = 11.91$, $p < 0.005$; time $F(1,29) = 2.69$, $p < 0.0001$)). Thus, D1/5 KO mice exhibit intact memory acquisition and consolidation of a weak contextual fear paradigm, however these mice display reduced freezing levels during an extinction session. Previous research has shown that stronger training in contextual fear can overcome hippocampal lesions and in order to test if stronger training would ameliorate freezing deficits mice were trained on a single 1 mA contextual fear-training paradigm (Wiltgen, Sanders et al. 2006). D1/5 KO mice exhibit freezing levels during training and test, which are not significantly different from control animals (Fig. 5-3). Moreover, when animals are trained and tested on the third paradigm, (three shocks, 0.75 mA) D1/5 KO freezing levels were not significantly different from flx control animals (Fig. 5-4). Furthermore, the same group was given a 15 minute extinction session, again the freezing levels between D1/5 KO and flx controls were not significantly different (Fig. 5-5). When given a 1 Hr short-term memory (STM) test after a three shock training paradigm, D5 KO animals do not differ in freezing as compared to controls (Fig. 5-6). These data suggest that D1/5Rs are not required for intact contextual fear memory acquisition and consolidation, however D1/5Rs are necessary during the extinction of a weak contextual fear-conditioning paradigm (Fig. 5-2).

D1/5Rs are Not Necessary in the Maintenance of Conditioned Fear Memory

Previous reports have suggested that D1/5Rs are necessary for the maintenance of hippocampal dependent memory (Rossato, Bevilaqua et al. 2009). In order to test this claim, D1/5 mice were trained on a three shock, 0.75 mA, contextual fear training paradigm. D1/5 KO mice exhibited similar freezing levels during training. Mice were tested in the same training context 7 days later and freezing levels during this test did not significantly differ from control mice (Fig. 5-7). These data suggest that D1/5Rs are not necessary for the maintenance of hippocampal dependent contextual fear conditioning.

Discussion

SCH 23390 studies have pointed to the D1R family in mediating the acquisition and consolidation of hippocampal dependent contextual learning and memory (Inoue, Izumi et al. 2000; Lemon and Manahan-Vaughan 2006; O'Carroll, Martin et al. 2006; Moncada and Viola 2007; Rossato, Bevilaqua et al. 2009). The data I present here is in opposition to this current view. D1/5 KO mice do not display significant deficits on contextual fear conditioning of a single 0.1 mA shock or three shock training paradigm, although there is a trend that is nearly significant in the single shock 0.1 mA test and future studies will be conducted using this same training and testing protocol (Fig. 5-1,3 and 4). Recent studies in ablated striatal spiny neurons in mice show that a 0.3 mA delayed fear training protocol results in LTM deficits. In the above studies I show that a single 0.5 mA contextual fear paradigm results in extinction deficits, which could be due to D1R deletion in the striatum and not due to altered hippocampal processing in the KO mice and it is not possible to rule out this possibility using these KO mice. These results provide evidence that D1/5Rs are not necessary for the acquisition or consolidation of contextual fear of stronger training paradigms, but other brain regions not necessarily involved in learning may be underlying these phenotypes.

The D1/5 KO studies presented above add evidence against SCH 23390 studies, there are three possible reasons as to why these data do not replicate. First,

SCH 233390 behavioral studies employ the use of subcutaneous, intraperitoneally (IP) or direct injections of drug into the brain region of interest. Subcutaneous and IP injections results in SCH 23390 spread throughout the body, including brain regions outside of the hippocampus. Studies utilizing cannulation and injection into brain regions of interest, cannot assure that SCH 23390 spread does not interact with other surrounding regions, such as the amygdala. Thus, behavioral results are likely to be non-specific to the brain region of interest. Second, SCH 23390 interacts with serotonergic receptors, which can alter plasticity in area CA1 of the hippocampus, resulting in non-specific receptor binding (Shakesby, Anwyl et al. 2002; Kojima, Matsumoto et al. 2003; Ryan, Anwyl et al. 2008; Zarrindast, Honardar et al. 2011). As a result, SCH 23390 may result in behavioral changes that are not specific to the D1R family. Third, SCH 23390 studies cannot confirm to what extent D1/5Rs are being antagonized. My data shows the extent to which D1Rs and D5Rs are deleted in the hippocampus (Fig. 2-8), while SCH 23390 studies to not quantify the amount of receptors blocked. Therefore, SCH 23390 studies do not provide adequate evidence that the D1R family is being blocked optimally.

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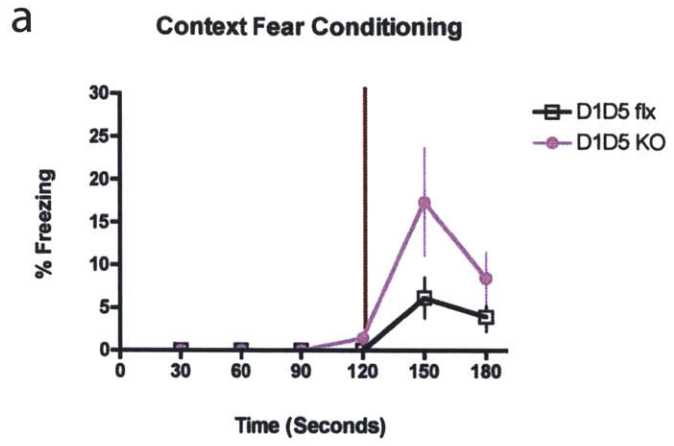
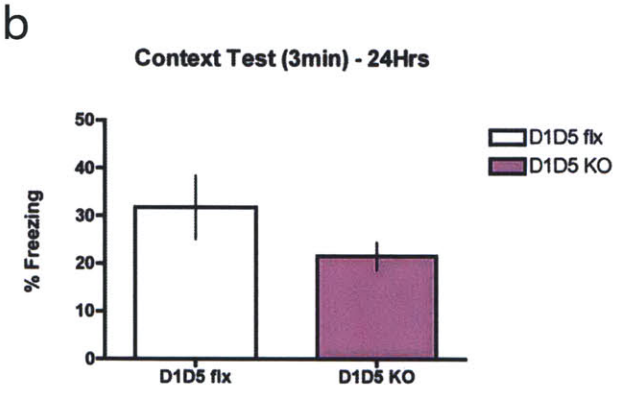
Figure 5-1

Single Shock (0.5 mA) Contextual Fear Conditioning - D1/5 Line, Long Term Memory Test





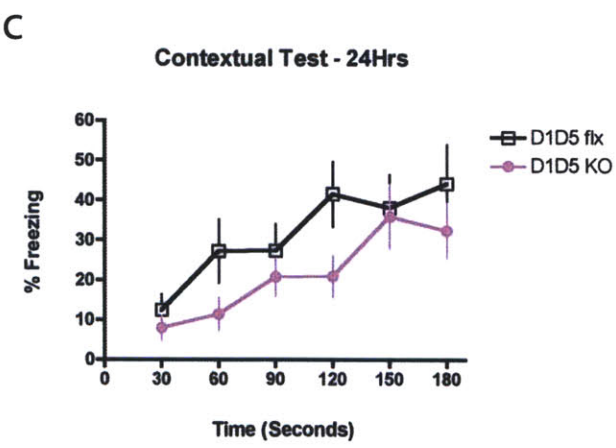


Figure 5-2

Single Shock (0.5 mA) Contextual Fear Conditioning - D1/5 Line, Extinction of Long Term Memory

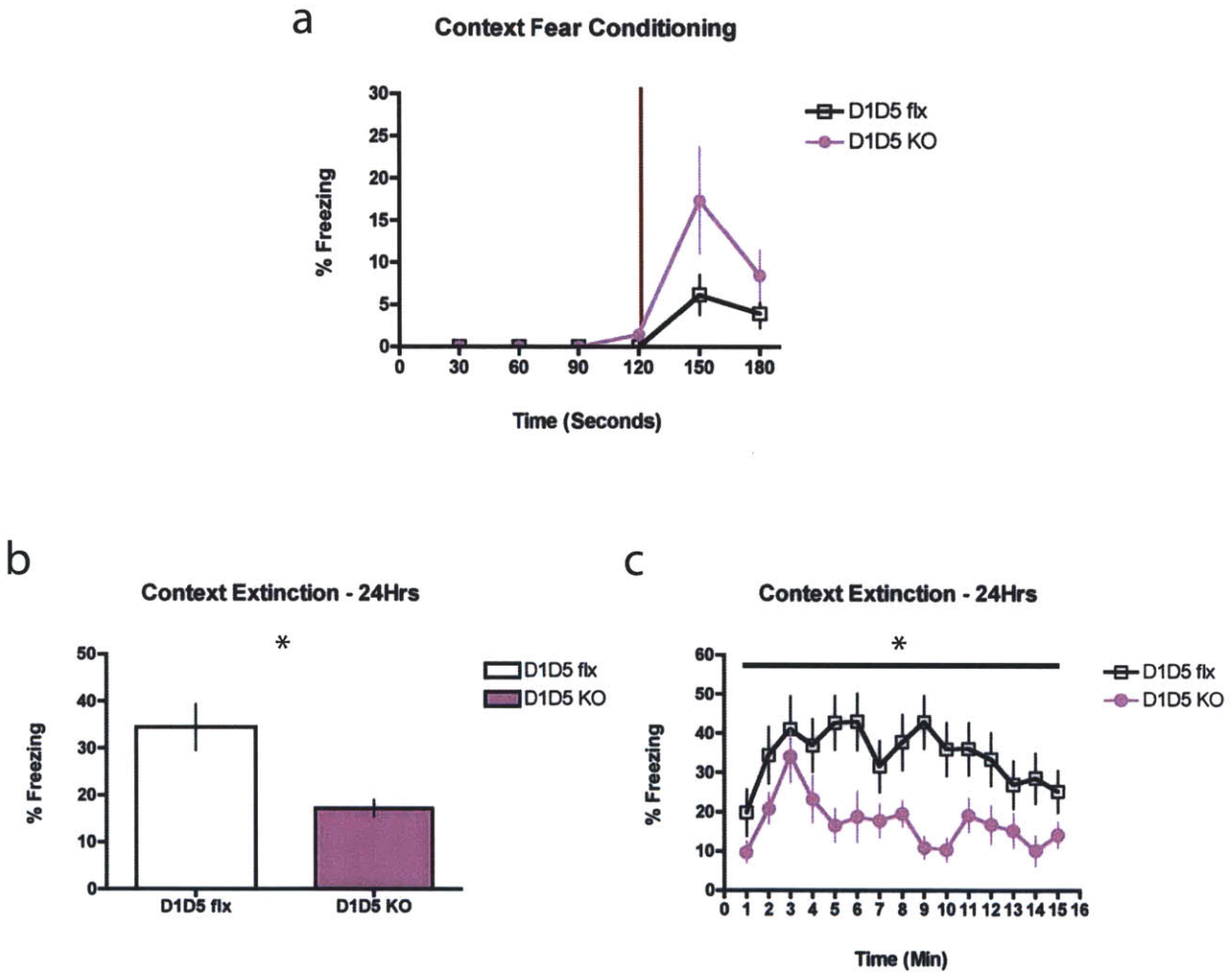
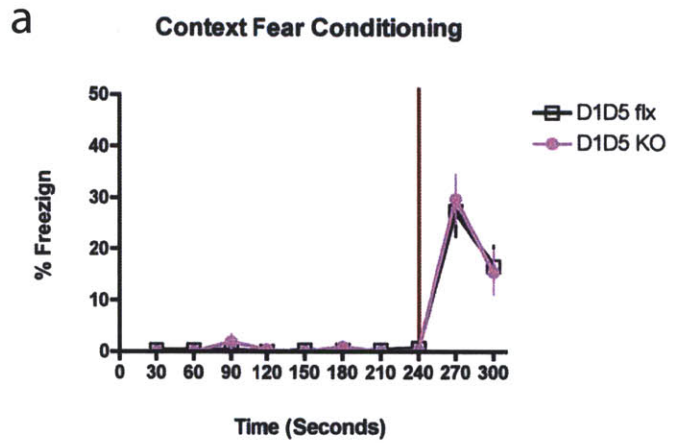
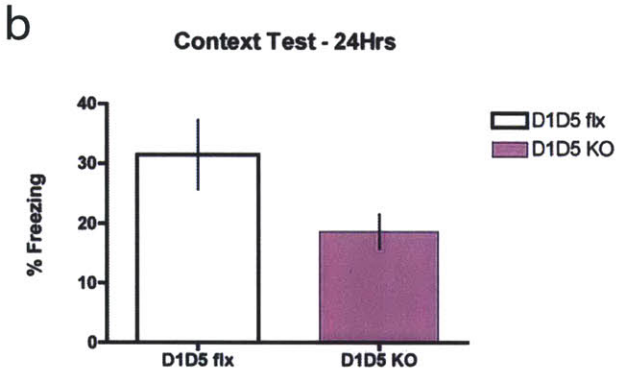


Figure 5-3

Single Shock (1 mA) Contextual Fear Conditioning - D1/5 Line, Long Term Memory Test





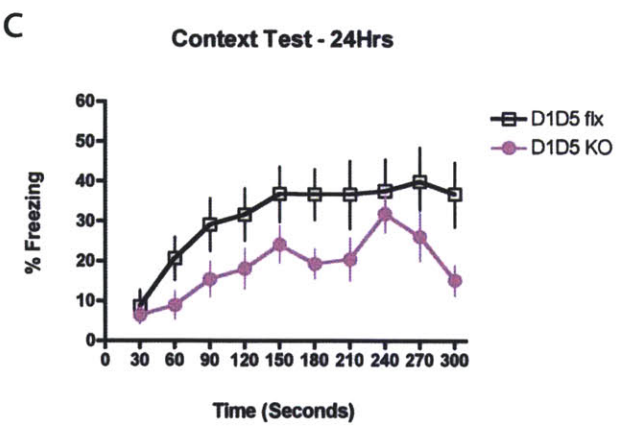
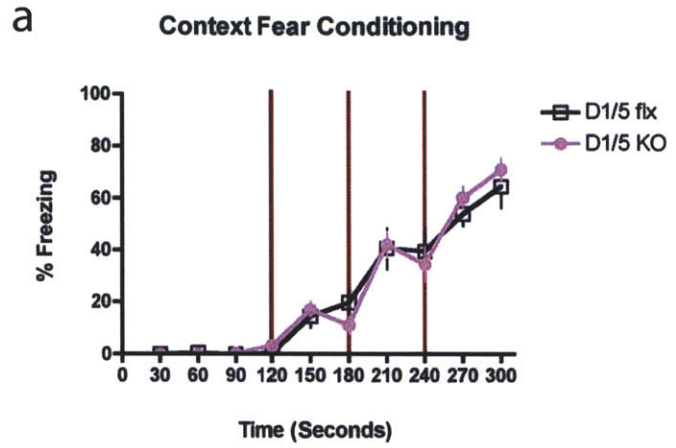
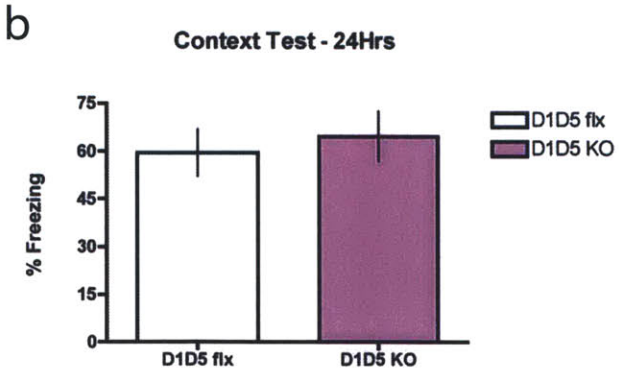


Figure 5-4

Three Shock Contextual Fear Conditioning - D1/5 Line, Long Term Memory Test





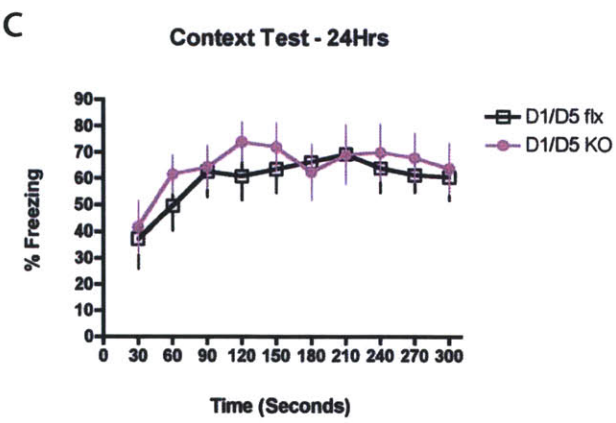
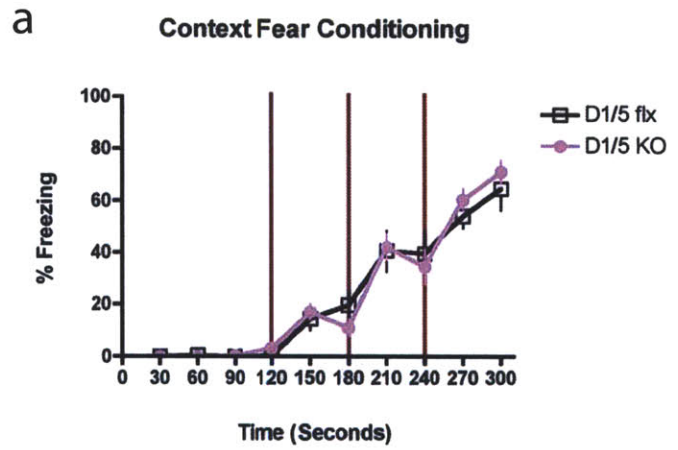
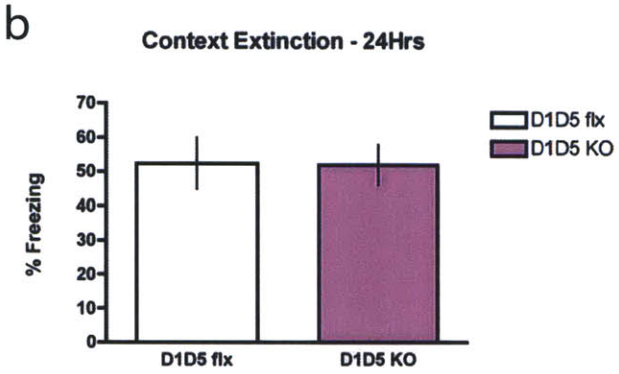


Figure 5-5

Three Shock Contextual Fear Conditioning - D1/5 Line, Extinction of Long Term Memory





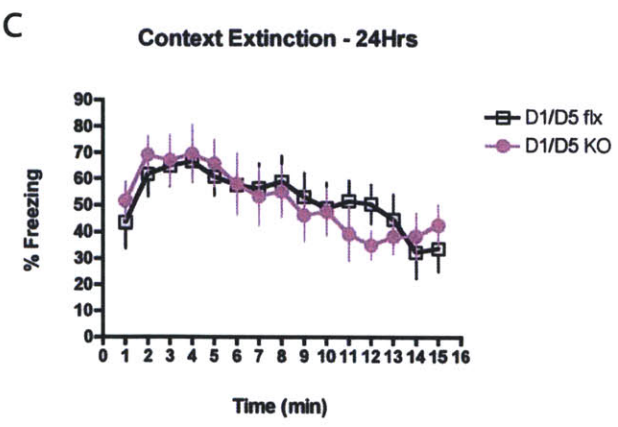
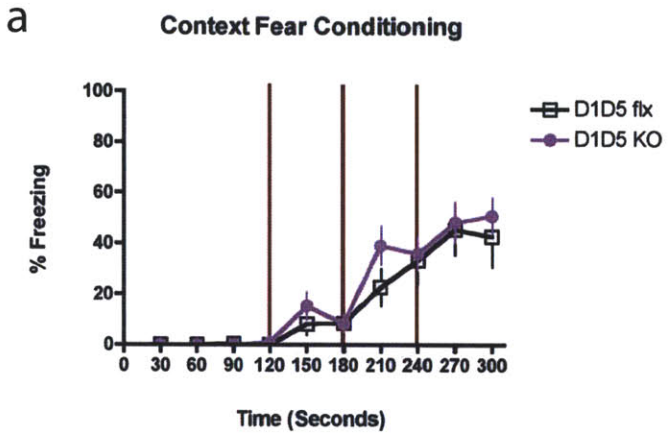
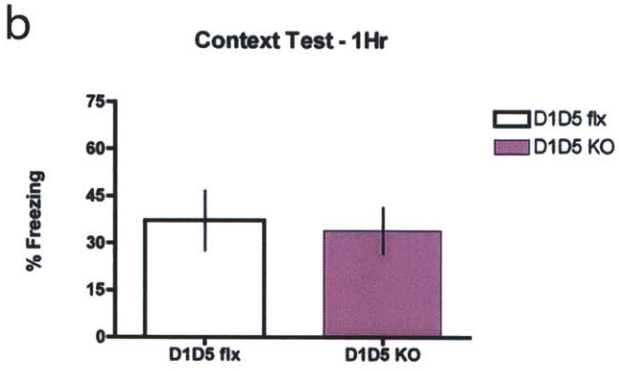


Figure 5-6

Three Shock Contextual Fear Conditioning - D1/5 Line, Short Term Memory Test





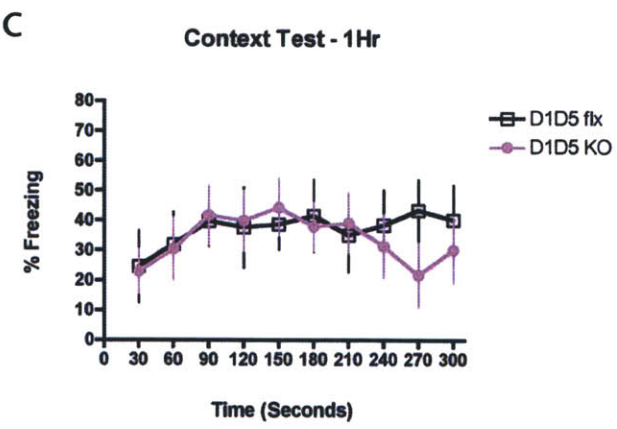
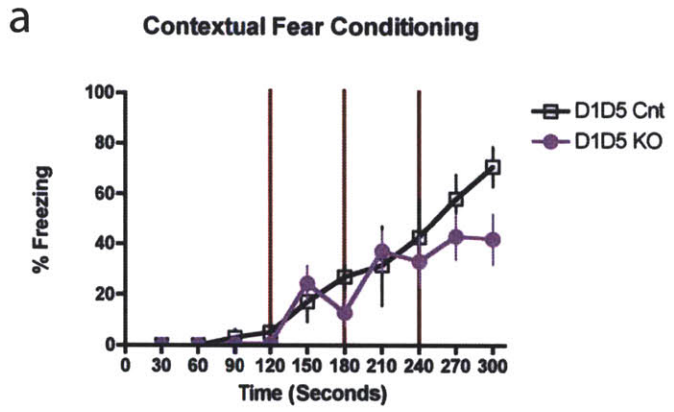


Figure 5-7

Three Shock Contextual Fear Conditioning - D1/5 Line, Memory Maintenance Test



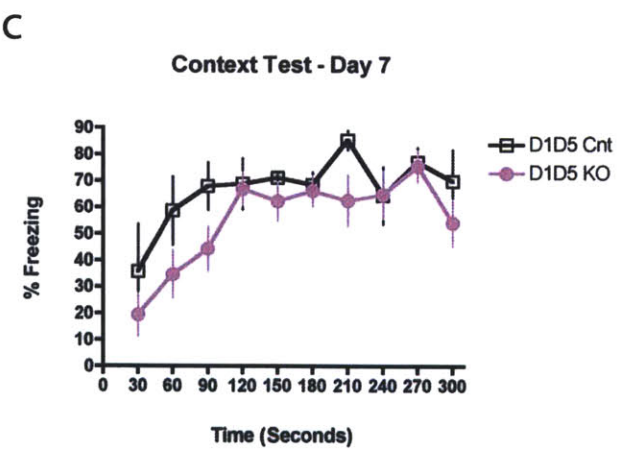


Figure 5-1. Single Shock (0.5 mA) Contextual Fear Conditioning – D1/5 Line, Long Term Memory Test

(a) Context Fear Conditioning. Single shock, 0.5 mA, context fear paradigm. D1/5 KO mice (n = 11) and D1/5 flx (n = 10).

(b) 24 Hr Context Test. 3 min. test 24 hours post training. D1/5 KO (n = 11) and D1/5 flx (n = 10).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-2. Single Shock (0.5 mA) Contextual Fear Conditioning – D1/5 Line, Extinction of Long Term Memory Test

(a) Context Fear Conditioning. Single shock, 0.5 mA, context fear paradigm. D1/5 KO mice (n = 11) and D1/5 flx (n = 10).

(b) 24 Hr Context Test. 15 min. extinction session test 24 hours post training. D1/5 KO mice (n = 11) exhibit significantly less freezing ($p < 0.005$) during extinction as compared to D1/5 flx (n = 10).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-3. Single Shock (1 mA) Contextual Fear Conditioning – D1/5 Line, Long Term Memory Test

(a) Context Fear Conditioning. Single shock, 1 mA, context fear paradigm. D1/5 KO mice (n = 6) and D1/5 flx (n = 8).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1/5 KO (n = 6) and D1/5 flx (n = 8).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-4. Three Shock Contextual Fear Conditioning – D1/5 Line, Long Term Memory Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D1/5 KO mice (n =11) and D1/5 flx (n = 9).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1/5 KO (n = 11) and D1/5 flx (n = 9).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-5. Three Shock Contextual Fear Conditioning – D1/5 Line, Extinction of Long Term Memory

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D1/5 KO mice (n =11) and D1/5 flx (n = 9).

(b) 24 Hr Context Test. 15 min. extinction session 24 hours post training. D1/5 KO (n = 11) and D1/5 flx (n = 9).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-6. Three Shock Contextual Fear Conditioning – D1/5 Line, Short Term Memory Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D1/5 KO mice (n = 9) and D1/5 flx (n = 9).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1/5 KO (n = 9) and D1/5 flx (n = 9).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Figure 5-7. Three Shock Contextual Fear Conditioning – D1/5 Line, Memory Maintenance Test

(a) Context Fear Conditioning. Three shocks (0.75 mA) context fear paradigm. D1/5 KO mice (n = 7) and D1/5 flx (n = 4).

(b) 24 Hr Context Test. 5 min. test 24 hours post training. D1/5 KO (n = 7) and D1/5 flx (n = 4).

(c) 24 Hr Context Test. Freezing timeline from same data as in (b).

Data are presented as mean +/- SEM.

Chapter 6

The Role of D1Rs and D5Rs in Spatial Navigation

Abstract

The role of the hippocampus in spatial navigation is well known. Global KO studies of D1 -/- mice or D5 -/- trained on the Morris water maze show that D1Rs are necessary for spatial learning, but D5Rs are not. Due to global deletion of the D1R it is unknown if the deficits are due to hippocampal processing or due to the deletion of the D1R in other brain regions known to be important in spatial navigation, such as the basal ganglia and cortical regions. Here, D1 KO, D5 KO and D1/5 KO mice are trained on a Morris water maze task and subsequently tested. In addition, mice are given a reversal training paradigm to test the role of D1Rs and D5Rs in preservative behavior. The data shows that D1 KO animals display significant latency deficits during training but show intact recall of spatial memory. When training on a reversal task, the latency deficits are greater than during regular training. Moreover, D1 KO animals exhibit deficits in spatial memory recall when given a probe trial test during reversal. In contrast to the D1 KO animals, D5 KO mice do not exhibit deficits in the acquisition, consolidation or expression spatial learning. During reversal D5 KO animals are also no different when compared to control littermate. When D1/5 KO animals are trained on the Morris water maze, these mice display latency deficits during training, similar to that of the D1 KO animals. Interestingly, D1/5 KO animals display spatial memory deficits when given a probe test during regular training, which is not seen in either the D1 KO or D5 KO lines. During reversal training, D1/5 KO

animals display latency deficits and also exhibit deficits in spatial memory recall, similar to that of the D1 KO animals. These data suggest that D1Rs are important and mediate, to an extent, the acquisition, consolidation and expression of spatial memory, while the D5Rs are not necessary. Furthermore, these data suggest that D5Rs function in spatial navigation when D1Rs are deleted, however to a minimal affect.

Introduction

Richard Morris showed that the hippocampus is necessary for spatial navigation in rodents (Morris, Garrud et al. 1982). Furthermore, he showed, for the first time, that blockade of NMDARs in and around the hippocampus leads to deficits in spatial learning and memory (Morris, Anderson et al. 1986). These data provided evidence that spatial learning requires NMDAR mediated plasticity in the hippocampus. With the advent of conditional KO mice, it was shown that NMDARs, specifically the NR1 subunit of the NMDAR, in area CA1 of the hippocampus is necessary for spatial learning (Tsien, Huerta et al. 1996). Recently, conditional KO mice with NMDAR deletion in area CA3 of the hippocampus also exhibit deficits in spatial learning in the context of pattern completion and one-trial learning (Nakazawa, Quirk et al. 2002; Nakazawa, Sun et al. 2003). In addition to the necessity of the NMDAR in hippocampal spatial navigation, the role of DA on hippocampal dependent spatial learning has also been investigated (Smith, Striplin et al. 1998; El-Ghundi, Fletcher et al. 1999; Holmes, Hollon et al. 2001; O'Carroll and Morris 2004; O'Carroll, Martin et al. 2006; Granado, Ortiz et al. 2007). As with the initial NMDAR studies, pharmacological inactivation of D1/5Rs, using SCH 23390, was used to study the role D1/5Rs in spatial navigation (O'Carroll, Martin et al. 2006). The author utilized a delayed match to place watermaze task and concluded that D1/5Rs are necessary for the persistence of 1 trial hippocampal memory trace. However,

pharmacological studies witch SCH 23390 cannot differentiate D1Rs from D5Rs, and given the potential differences in function of these receptors, it is unknown as to which receptor mediates spatial learning (Gines, Hillion et al. 2000; Liu, Wan et al. 2000; Lee, Xue et al. 2002; Sahu, Tyeryar et al. 2009). Furthermore, it is near impossible to estimate the effective hippocampal concentration when drug is delivered by acute intrahippocampal infusion (O'Carroll, Martin et al. 2006). In order to circumvent the inability to differentiate D1Rs from D5Rs, two global KO mice, the D1 -/- and D5 -/- global KOs, have been created and used to study spatial navigation (Smith, Striplin et al. 1998; El-Ghundi, Fletcher et al. 1999; Holmes, Hollon et al. 2001; Granado, Ortiz et al. 2007). Although global D1 -/- and D5 -/- KO animals allow for the differentiation of D1Rs from D5Rs, these mice do not allow for spatially restricted deletion of these receptors. Given the role of D1Rs in the basal ganglia for intact motor activity, the deficits shown in the D1 -/- KO studies may in fact be due to deficits in motor activity rather than deficits in spatial navigation mediated by the hippocampus (Xu, Moratalla et al. 1994). Moreover, cortical lesions also affect spatial navigation, thus D1 -/- global KO deficits may be due to cortical disruption of DA mediated activation rather than DA mediated activation within the hippocampus (Whishaw and Kolb 1984). Thus, we see that pharmacological studies, which can be region specific, do not delineate the functions of D1Rs from D5Rs and global KO studies, which can differentiate D1Rs from D5Rs, cannot offer region specificity. As a result, the role of the D1R or the D5R in hippocampal spatial learning is unknown.

Conditional KO mice offer both region specificity as well as the ability to differentiate the functions of the D1R and D5R. As with the NMDAR conditional KO animals, we are able to offer hippocampal subregion precision in our D1, D5, and D1/5 KO animals. We show that the D1 KO animal, D5 KO animal and combination of both, the D1/5 KO animal, exhibit distinct behavioral phenotypes when tested on the Morris water maze.

Methods and Materials

Mice were subjected to a reference memory version of the Morris water maze, where they utilized allocentric cues in learning and recalling spatial memories (Morris, Garrud et al. 1982). D1 KO (n = 10), D1 flx (n = 11), D5 KO (n = 10), D5 flx (n = 9), D1/5 KO (n = 9) and D1/5 flx (n = 8) mice, with ages between 30-40 weeks. Each animal was given four training trials per day, for 11 days, with ~ 1 hour in between each training trial. Animals were required to find a 12cm-hidden platform below 2cm of opaque orange pool water in a 1.6m diameter pool. Data was collected using ImageWater 2020 software. Each training trial lasted a maximum of 60 seconds and if the animal did not find the platform, he was laced on the platform for 15 seconds. Animals were trained for 10 days and received a probe test, which consisted of 60-second test with a removed hidden platform, on days 6 and 11 (prior to the training trial for that day). After the second pilot test the hidden platform was placed back into the pool but positioned adjacent to the original location during the initial training. Mice were retrained following the same 10 day protocol with two probe trials on days 6 and 11.

Results

The Morris Water Maze was utilized to test the role of D1Rs and D5Rs in hippocampal dependent spatial navigation. All mice received 10 days of training and a spatial memory probe trial on days 6 and 11. After this initial training, all mice received reversal (platform placement opposite to original position) training for 10 days with probes on days 6 and 11. D1 KO mice exhibit significant latency deficits during the initial training but do not show deficits in the probe trials during regular training (Fig. 6-1a; two-way ANOVA (genotype x time) $F(1,9) = 0.39$, $p = 0.94$; genotype $F(1,9) = 9.38$, $p < 0.01$; time $F(1,9) = 23.53$, $p < 0.0001$). Additionally, D1 KO mice show significant latency deficits during the reversal training and a reversal probe deficit on day 11 (Fig. 6-2a; two-way ANOVA (genotype x time) $F(1,9) = 0.42$, $p = 0.42$; genotype $F(1,9) = 7.76$, $p < 0.05$; time $F(1,9) = 21.12$, $p < 0.0001$, c; $p < 0.05$, compare to flx, Student's t test). D5 KO mice show no difference during regular training, probes, reversal training or reversal probe (Fig. 6-3 and 4). D1/5 KO display significant latency deficits as well as deficits in the probe trial on the day 11 probe during regular training (Fig. 6-5a; two-way ANOVA (genotype x time) $F(1,9) = 1.12$, $p = 0.36$; genotype $F(1,9) = 10.45$, $p < 0.001$; time $F(1,9) = 28.24$, $p < 0.0001$, c; $p = 0.01$, compare to flx, Student's t test). These mice also show a reversal latency deficits as well as reversal probe deficit on day 11 (Fig. 6-6a; two-way ANOVA (genotype x time)

$F(1,9) = 0.67$, $p = 0.74$; genotype $F(1,9) = 5.49$, $p < 0.05$; time $F(1,9) = 18.53$, $p < 0.0001$, c; $p = 0.01$, compare to flx, Student's t test). D1/D5 KO mice exhibit impairments in spatial memory recall during regular training, while D1 KO and D5 KO mice show no differences compared to controls. However, during the reversal training and spatial memory tests, both the D1 KO and D1/5 KO display deficits in spatial memory recall, as shown by less time spent in the correct quadrant on the second probe. These results suggest that the D5R is able to compensate for some of the deficits of the D1R KO in spatial learning during regular training. Yet, the D5 KO is not able to compensate during the reversal training and spatial memory test as both the D1 KO and D1/5 KO exhibit similar deficits in spatial memory. Moreover, the D5R is dispensable in spatial navigation as the D1R is sufficient to mediate the Morris watermaze task.

Discussion

D1 KO mice exhibit deficits in latency during spatial training, however the recall of spatial memory is intact. The latency deficits during reversal, however, are greater and on the last day of spatial memory recall these animals spend significantly less time in the correct quadrant when compared to control littermates (Fig. 6-1 and 2). Although D1 KO animals display spatial memory recall deficits during the last reversal probe, these animals still spend a significantly greater period of time in the correct quadrant than other quadrants, but not to the same extent as control animals (Fig. 6-2c). D1 KOs are not impaired in spatial memory but are impaired when the task is switched, such as in placing the platform in the opposite quadrant (Fig. 6-2). These spatial memory recall deficit during the last probe of the reversal task suggests that there may be preservative errors in these mice, such that they continue to go to the original quadrant where the platform was located during initial training. This is not the case, as animals do not spend more time in the original training quadrant during reversal training or during probe tests (Fig. 6-2b,c). Deficits in the water maze may be due to motor inability as the D1 KO animals do exhibit decreased D1R expression in the dorsal-medial caudal basal ganglia (Fig. 2-6a,b). However, when tested on the rotarod and when the total distance during an open field test is measured, there are no significant differences between D1 KO animals and control littermates (Fig. 3-3 thru 5). Moreover, these animals do not utilize

different strategies in finding the hidden platform, such as thigmotaxis, which is also true for the mice (Data not shown). This provides evidence that motor deficits are not the underlying problem in this task. Moreover, D1 KO animals do not display the same degree of deficits as seen with the D1 -/- global KO animals, suggesting that D1Rs in the hippocampus are not necessary for spatial learning per se, but are necessary for enhancing the degree of memory recall (Granado, Ortiz et al. 2007). Thus, D1Rs in the hippocampus are sufficient to enhance the degree of learning, although they are not necessary for spatial learning.

The D1/5 KO animal exhibits deficits in spatial memory recall that occurs earlier in training than in the D1 KO animals (Fig. 6-5). The deletion of the D5R adds to the deficits in spatial memory, this provides evidence that D5Rs can ameliorate some of the deficits observed in the D1 KO animal, even though D5 KO animals exhibit no deficits in spatial learning and memory (Fig. 6-3 and 4). This finding runs in opposition to the phenotypes observed in contextual fear conditioning, where D1 KO animals display reduced freezing during training on a strong training paradigm, but when the D5R is also deleted (D1/5 KO animals) the deficits are fully rescued (Fig. 5-4). Thus, D5Rs are not able to substitute when D1Rs are deleted. These results show that D1Rs and D5Rs have differing functions in hippocampal processing and that these function change depending

on which hippocampal task the animal is undergoing, such as in classical conditioning versus spatial learning.

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Figure 6-1

Spatial Learning and Memory, D1 Line
Morris Water Maze

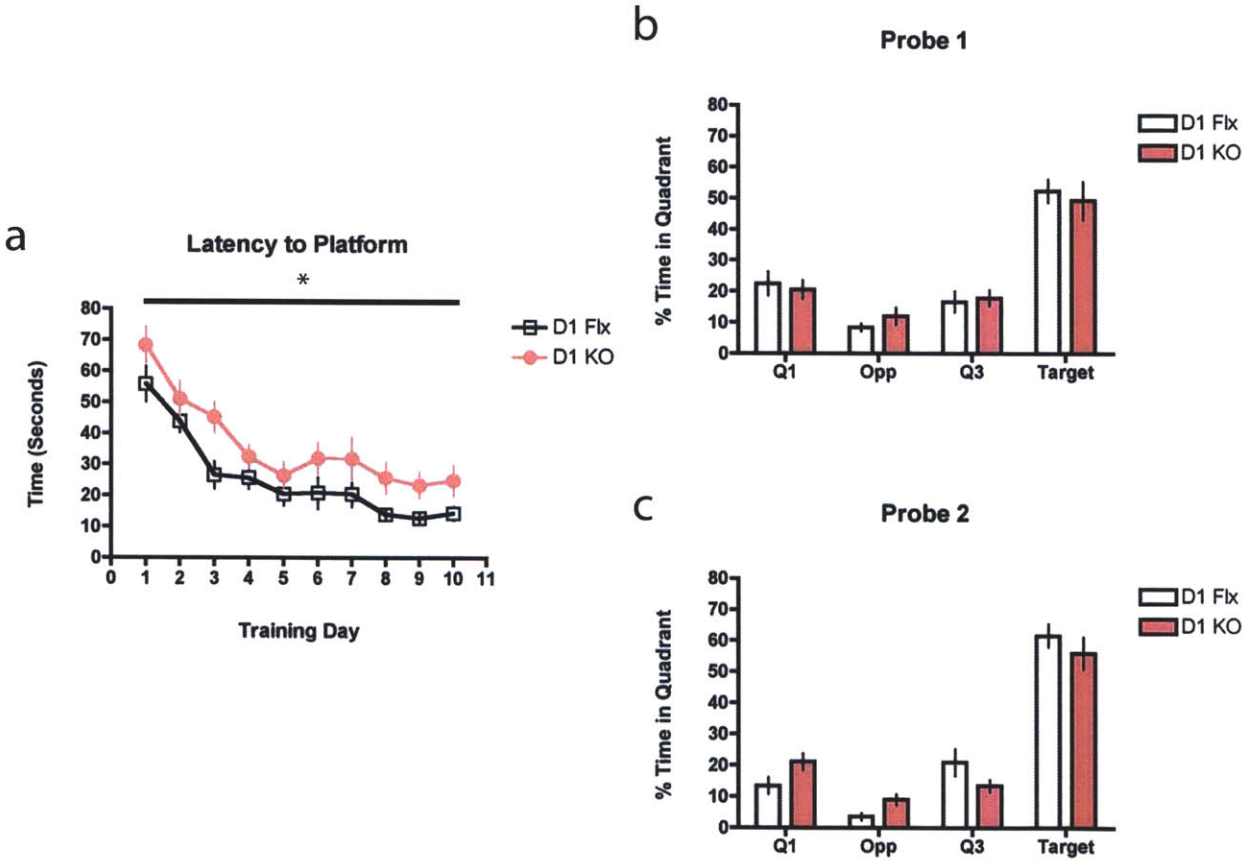

Figure 6-2

Spatial Learning and Memory, D1 Line
Morris Water Maze Reverse Training

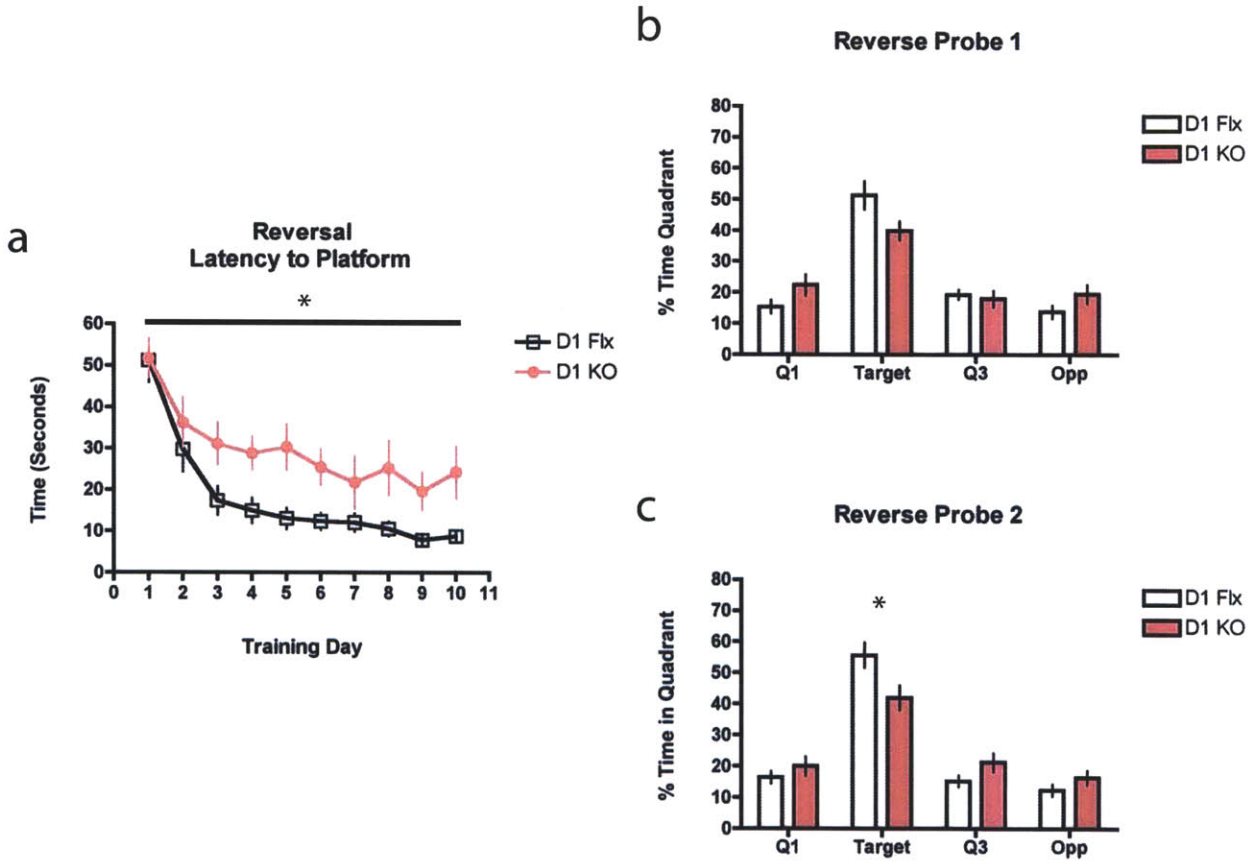

Figure 6-3

Spatial Learning and Memory, D5 Line
Morris Water Maze

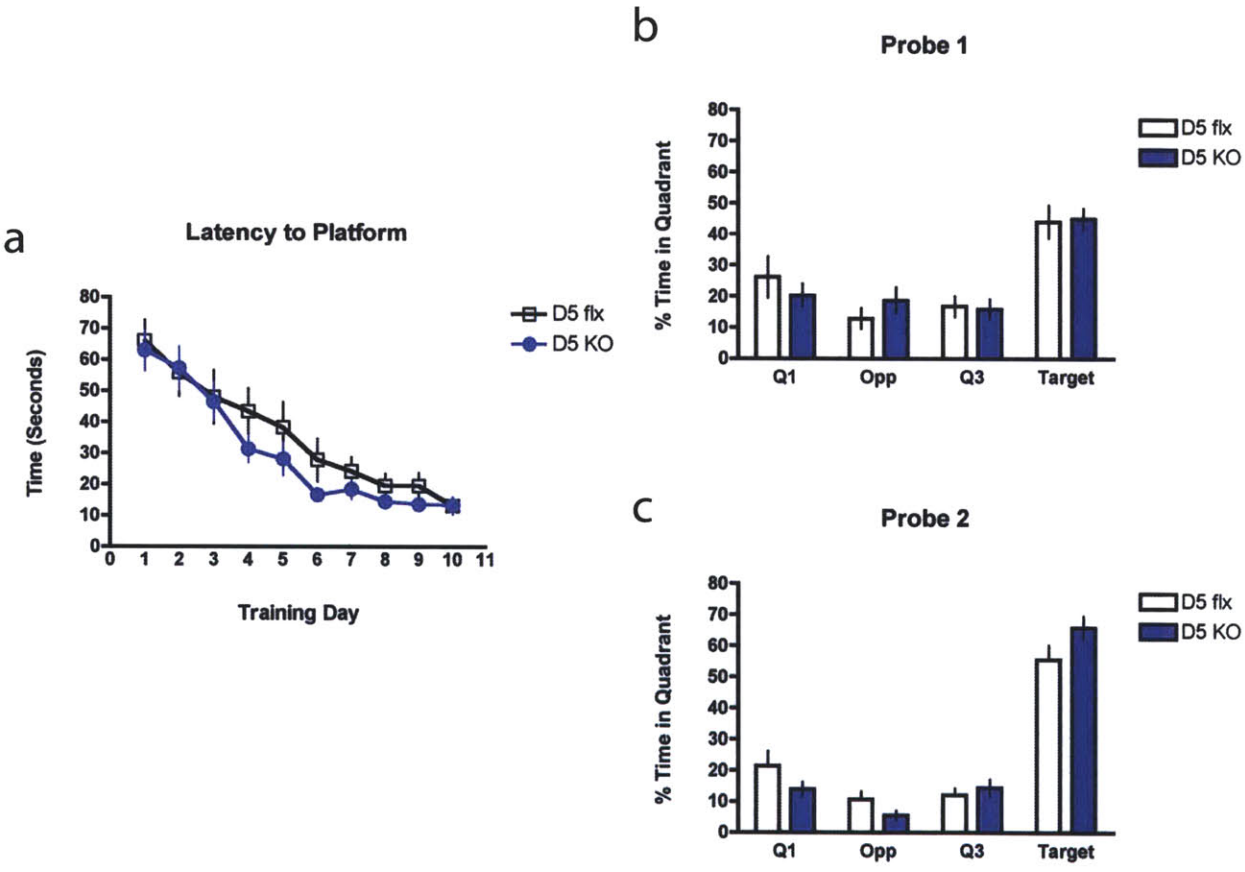

Figure 6-4

Spatial Learning and Memory, D5 Line
Morris Water Maze Reverse Training

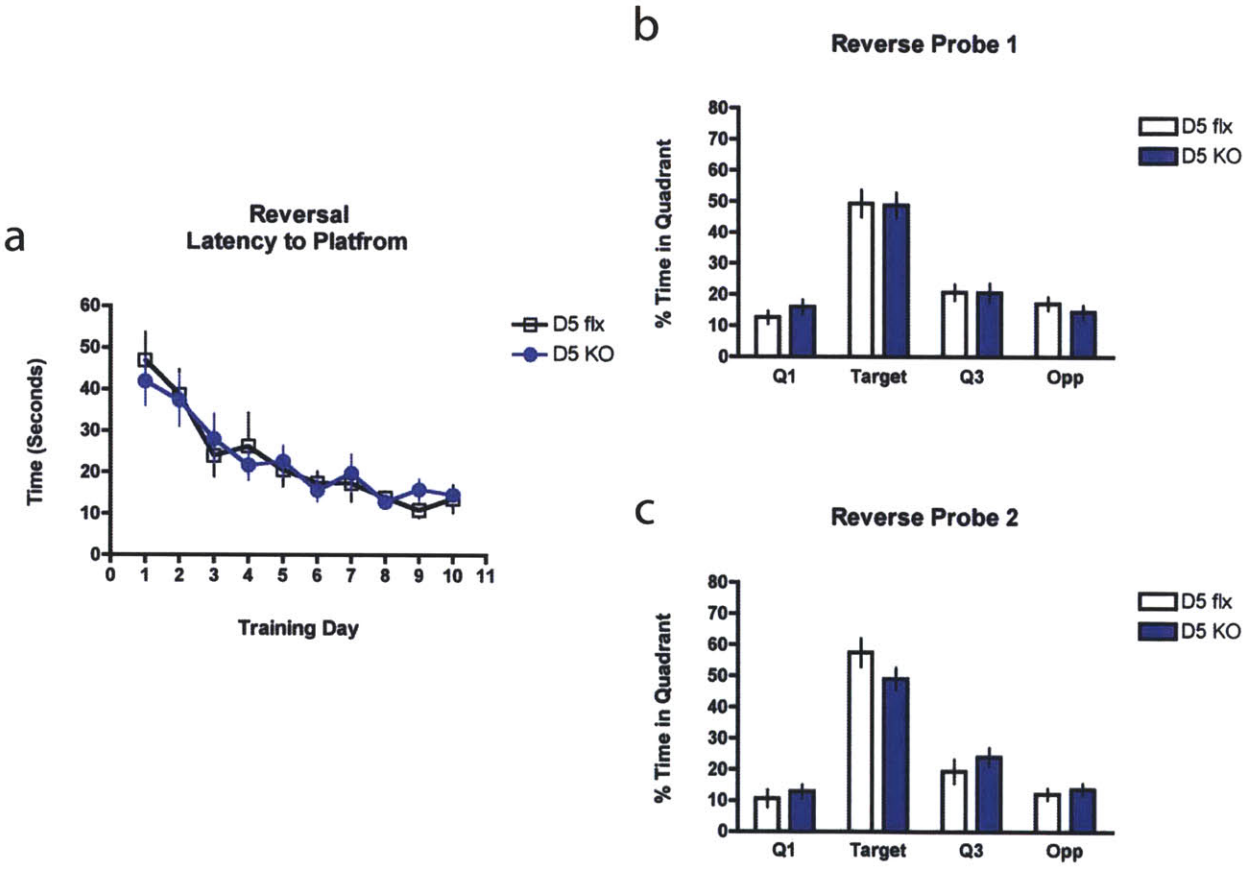

Figure 6-5

Spatial Learning and Memory, D1/5 Line
Morris Water Maze

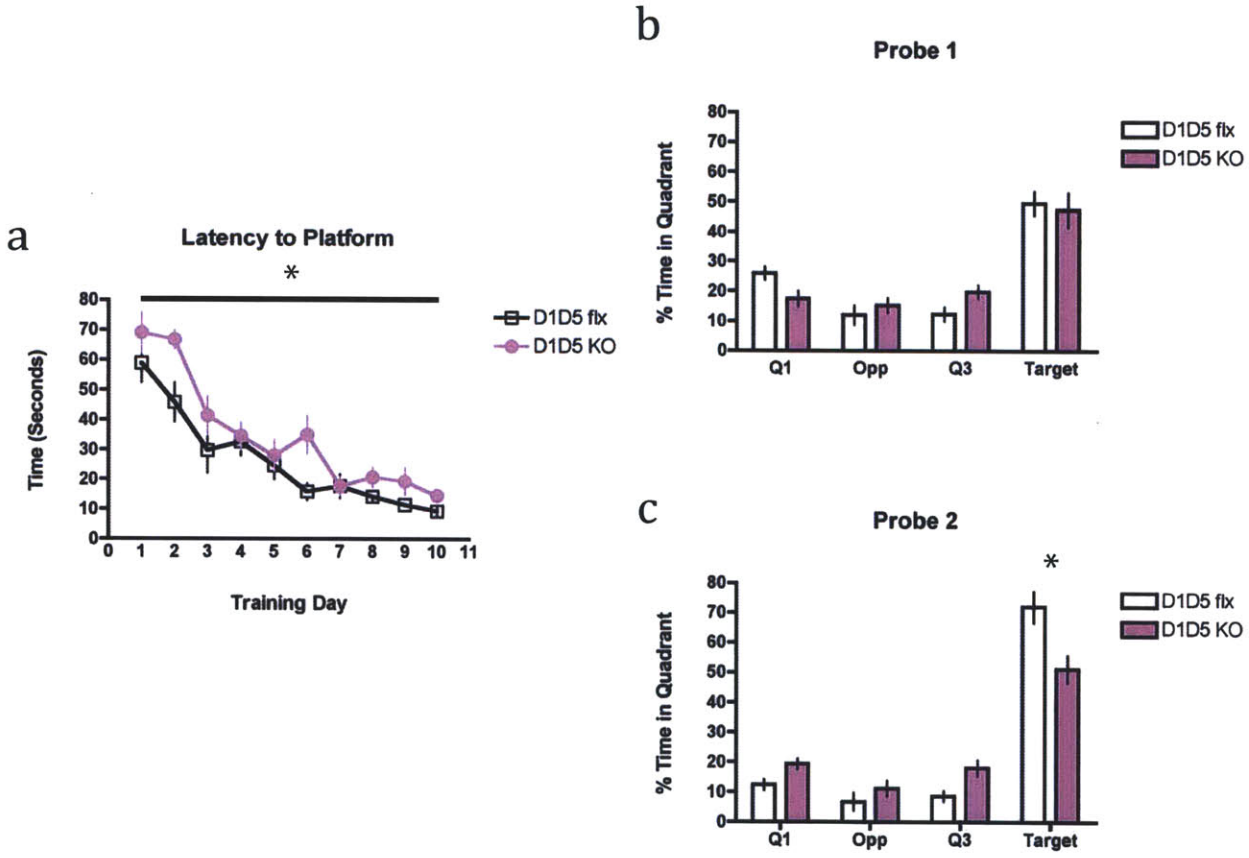

Figure 6-6

Spatial Learning and Memory, D1/5 Line
Morris Water Maze Reverse Training

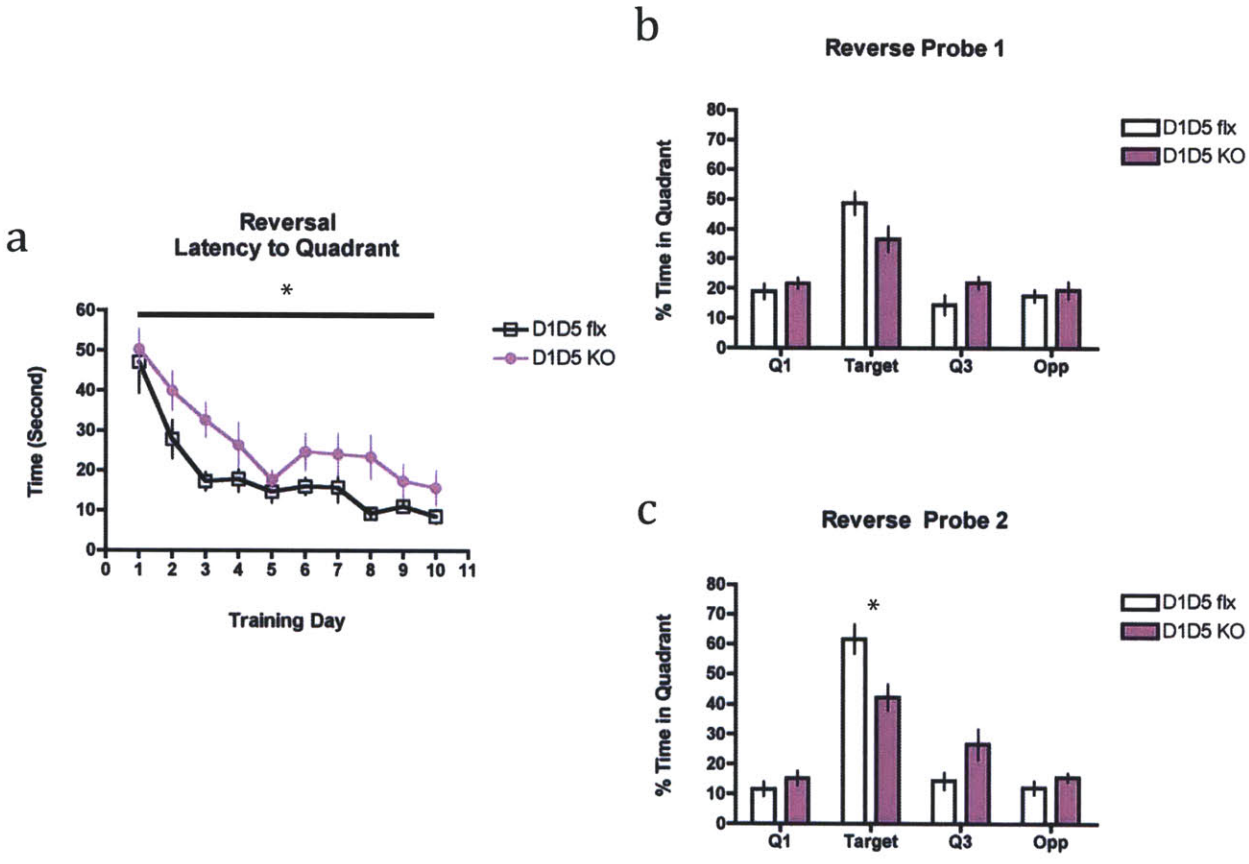

Figure 6-1. Spatial Learning and Memory, D1 Line, Morris Water Maze

(a) Morris Water Maze Training. Latency during training from days 1 through 11. D1 KO (n = 10) mice exhibit significantly greater latency ($p < 0.01$) times during training as compared to D1 flx (n = 11).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D1 KO (n = 10) and D1 flx (n = 11).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D1 KO (n = 10) and D1 flx (n = 11).

Figure 6-2. Spatial Learning and Memory, D1 Line, Morris Water Maze Reversal Training

(a) Morris Water Maze Training. Latency during reversal training from days 1 through 11. D1 KO (n = 10) mice exhibit significantly greater latency ($p < 0.05$) times during training as compared to D1 flx (n = 11).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D1 KO (n = 10) and D1 flx (n = 11).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D1 KO mice (n = 10) spend significantly less time in the correct quadrant ($p < 0.05$) during the second probe trial as compared to D1 flx (n = 11).

Figure 6-3. Spatial Learning and Memory, D5 Line, Morris Water Maze

(a) Morris Water Maze Training. Latency during training from days 1 through 11. D5 KO (n = 10) and D5 flx (n = 9).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D5 KO (n = 10) and D5 flx (n = 9).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D5 KO (n = 10) and D5 flx (n = 9).

Figure 6-4. Spatial Learning and Memory, D5 Line, Morris Water Maze Reversal Training

(a) Morris Water Maze Training. Latency during reversal training from days 1 through 11. D5 KO (n = 10) and D5 flx (n = 9).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D5 KO (n = 10) and D5 flx (n = 9).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D5 KO (n = 10) and D5 flx (n = 9).

Figure 6-5. Spatial Learning and Memory, D1/5 Line, Morris Water Maze

(a) Morris Water Maze Training. Latency during training from days 1 through 11. D1/5 KO (n = 9) mice exhibit significantly greater latency ($p < 0.05$) times during training as compared to D1/5 flx (n = 8).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D1/5 KO (n = 9) and D1/5 flx (n = 8).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D1/5 KO mice (n = 9) spend significantly less time in the correct quadrant ($p = 0.01$) during the second probe trial as compared to D1/5 flx (n = 8).

Figure 6-6. Spatial Learning and Memory, D1/5 Line, Morris Water Maze Reversal Training

(a) Morris Water Maze Training. Latency during reversal training from days 1 through 11. D1/5 KO (n = 9) mice exhibit significantly greater latency ($p < 0.05$) times during training as compared to D1/5 flx (n = 8).

(b) Spatial Memory Probe Trial 1. 60 second probe trial 6 days post training. D1/5 KO (n = 9) and D1/5 flx (n = 8).

(c) Spatial Memory Probe Trial 2. 60 second probe trial 11 days post training. D1/5 KO mice (n = 9) spend significantly less time in the correct quadrant ($p = 0.01$) during the second probe trial as compared to D1/5 flx (n = 8).

Data are presented as mean +/- SEM.

Experiments Conducted by Joshua Sariñana

Chapter 2

- Probe design in collaboration with Lisa Knopf (Figure 2-1)
- Quantification of in situ hybridization data (Figure 2-8)
- All *in-vivo* physiology experiments (Figure 2-11 and 12)

Chapter 3

- Hot plate sensitivity test (Figure 3-1)
- Elevated plus maze experiments in collaboration with Nirupama Yechoor (Figure 3-2)
- Open field experiments in collaboration with Nirupama Yechoor (Figure 3-3 and 4)
- Rotarod test collaboration with Nirupama Yechoor (Figure 3-5)
- All Delayed fear conditioning experiments (Figure 3-6 thru 8)

Chapter 4

- All contextual fear conditioning experiments (Figures 4-1 thru 5)

Chapter 5

- All contextual fear conditioning experiments (Figures 5-1 thru 7)

Chapter 6

- All watermaze experiments (Figures 6-1 thru 6-6)

Acknowledgements

The following individuals added significant work to my thesis project. What each individual added to the thesis is described below.

Name	Position	Project
Amy LeMessurier	Laboratory Technician	SCH and Veh Slice Physiology (See Fig. 2-9 and 10)
Lisa Knopf	Laboratory Technician	mRNA Probe Development and Synthesis (See Fig. 2-1)
Amanda Dellevigne	Laboratory Technician	Mouse Colony Maintenance
Nirupama Yechoor	UROP Student	Behavioral Battery Experiments (See Fig 3-2 through 5)
Wenjiang Yu	Laboratory Technician	In Situ Hybridization Experiments (See Fig. 2-2 through 7)
Jared Martin	Laboratory Technician	Mouse Colony Maintenance