A histone deacetylase inhibitor, trichostatin-A, induces odor preference memory extension and maintains enhanced AMPA receptor expression in the rat pup model

by

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ABSTRACT

Histone deacetylase (HDAC) plays a role in synaptic plasticity and long-term memory formation. I hypothesized that trichostatin-A (TSA), an HDAC inhibitor, would promote long-term odor preference memory and maintain enhanced GluA1 receptor levels that might support memory. I used an early odor preference learning model in neonate rat pups to test behavior and examine receptor protein expression. My behavioral studies showed that intrabulbar infusion of TSA, prior to pairing of the conditioned stimulus (peppermint odor) with the unconditioned stimulus (tactile stimulation), prolonged odor preference memory for at least nine days. Western blot analysis showed that GluA1 receptor membrane expression in the olfactory bulbs of TSA-treated pups was significantly increased at 48 h. Immunohistochemistry revealed significant increase of GluA1 expression in olfactory bulb glomeruli five days after training. These results support evidence for a relationship between enhanced GluA1 receptor expression and memory. These findings will permit further exploration of mechanisms which induce and maintain memories.

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5-HT	serotonin
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AR	adrenoceptor
β-ARs	β-adrenorceptors
BCA	bicinchoninic acid
BSA	bovine serum albumin
Ca^{2+}	calcium ion
cAMP	cyclicAMP
CBP	CREB binding protein
cilo	cilomilast
Cl^-	chloride ion
CAMKII	calmodulin dependent protein kinase II
CNS	central nervous system
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
CS	conditioned stimulus
DAB	diaminobenzidine
DM	dorsomedial
DMSO	dimethyl sulfoxide
DL	dorsolateral
ECL	enhanced chemiluminescent
EDTA	ethylenediaminetetraacetic acid
EPL	external plexiform layer
ET	external tufted cells
GCL	granule cell layer
GL	glomerular layer
h	hour
HAT	histone acetyltransferase
HDAC	histone deacetylase
H_2O_2	hydrogen peroxide
IPL	internal plexiform layer
Iso	isoproterenol
IHC	immunohistochemistry
JG	juxtaglomerular neuron
LC	locus coeruleus
LOT	lateral olfactory tract

LTP	long-term potentiation
MCL	mitral cell layer
M/T	mitral and tufted cells
Min	minute
Na^+	sodium ion
NaB	sodium butyrate
NE	norepinephrine
NMDA	N-methyl-D-aspartic acid
OB	olfactory bulb
OD	optical density
ONL	olfactory nerve layer
O/O	odor only
ORN	olfactory receptor neuron
O/S	odor + stroking
PND	post-natal day
PDE4	phosphodiesterase 4
PKA	protein kinase-A
PG	periglomerular cells
RMS	rostral migratory stream
SDS	Sodium dodecyl sulphate
Ser-133	Serine-133
SEL	subependymal layer
SA	short-axons cells
SVZ	subventricular zone
TBST	tris-buffered saline with tween
TSA	trichostatin- A
UCS	unconditioned stimulus

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CHAPTER 1- INTRODUCTION

1.1 Overview

Memory is a process in which information is encoded, stored and retrieved. It depends on multiple cellular and molecular mechanisms. There are several animal models for memory. The most explored simple model of memory is that in *Aplysia,* which was developed by Eric Kandel and his colleagues. Many other models have been developed to understand the behavioural, cellular and molecular mechanisms of memory. The research presented in this thesis involves a developmental mammalian model of learning.

The early odor preference learning model is a classical conditioning model in rat pups. During early life, rat pups need to recognize their mother's odor for survival. The association of the pup with their mother is critical to survival. They use odor recognition to maintain proximity to their dam. In this model, on post-natal day (PND 6) rat pups learn to prefer a novel odor by having it paired for 10 min with tactile stimulation (stroking) that mimics maternal care. This learning model gives 24 h long-term memory, but normally not longer than 24 h (Sullivan and Leon, 1986; Sullivan et al., 1986; McLean et al., 2005).

The purpose of this study is to establish a model of prolonged memory lasting beyond 24 h and to examine the potential mechanisms involved in memory extension. In this study, I try to extend olfactory preference memory by using histone deacetylase (HDAC) inhibition and I then establish a correlation between memory extension and receptor

expression related to synaptic plasticity in the olfactory bulb.

1.2 Olfactory bulb circuitry

Odorants are volatile compounds that enter the nose and bind to a receptor within the nasal epithelium. An odorant binding to a specific receptor is based on its structure (Reed, 1992). There are approximately 1000 types of odorant receptors (ORs) expressed on the dendrites of olfactory receptor neurons (ORNs) in the nasal epithelium of mice and rats (Buck and Axel, 1991; Ressler et al., 1993). Each ORN expresses a single type of OR out of the many possible types (Young and Trask, 2002). In the nasal epithelium, ORNs expressing homologous OR genes, send axons that converge and terminate within a few specific spherical glomeruli within the OB (Mombaerts et al., 1996).

The glomerulus is a structural unit in the OB that contains elements that relay the information from ORNs to second–order neurons: mitral, tufted and periglomerular cells in the OB. Several experiments (molecular genetics, electrophysiology, in-vivo imaging, behavioural experiments) showed that different odors are represented at specific topographic locations in the OB (Woo et al., 1987; Keller et al., 1998). For this reason, a given odor will activate a specific pattern of glomerular- related OB circuitry (Jourdan et al., 1980; Johnson and Leon, 2007).

1.2.1 Layers of the OB

The OB is a highly organized structure that consists of several distinct layers. From the most outer to inner, these layers are the olfactory nerve layer (ONL), glomerular layer

(GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GCL) and subependymal layer (SEL).

1.2.1.1 Olfactory nerve layer (ONL)

The ONL is the outermost layer of the OB. The axons of the olfactory neurons initially enter the ONL and terminate in the glomerular layer (Shipley and Ennis, 1996).

1.2.1.2 Glomerular layer (GL)

The GL is located near the surface of the olfactory bulb; deep to the ON layer. The GL is composed of spherical neuropil filled structures. Each spherical shaped structure has a diameter ranging from 80-160 µm, and approximately 3000 glomeruli are present in rat olfactory bulb (Meisami and Safari, 1981). A single glomerulus is surrounded by thousands of juxtaglomerular neurons (JG) and glial cells (O'Connor and Jacob, 2008). JG are a collective set of small neurons composed of periglomerular cells (PG), external tufted cells (ET) and short-axons cells (SA). Together, these JG cells form a complex interglomerular network (Pinching and Powell, 1971a).

PG cells are small (5-8µm) spherical cells that are the most abundant cells in the GL. These cells are either GABAergic, dopaminergic or both (Shipley and Ennis, 1996). A large population of PG dopaminergic cells are present in the GL whereas GABAergic PG cells represent approximately 20% of the PG cells (O'Connor and Jacob, 2008). PG cells receive excitatory input from ORN terminals and initiate inhibitory feedback from PG cells on to MC and ET cells. PG cells also receive monosynaptic excitatory input from ET cells and produce inhibitory feedback to MC cells (Pinching and Powell, 1971b).

ET cells are excitatory neurons and are the largest cells of the GL. They lie deep within the periglomerular region of the GL. ET cells have a single dendrite that extends into a glomerulus (Pinching and Powell, 1971b; Hayar et al., 2004a, 2004b). Electrophysiological studies have been shown that ET cells receive monosynaptic ORN input and produce excitatory feedback on to PG and SA cells (Hayar et al., 2004a; Liu and Shipley, 2008). ET cells can also excite MC cells directly (Najac et al., 2011).

SA cells are found frequently in the PG region. They are either GABAergic or dopaminergic in nature. They do not receive any direct input from ORN terminals but do receive input from other SA and ET cells (Hayar et al., 2004a). SA cells can extend multiple dendrites into the GL and form synapses with PG cells. SA cells play a vital role in the interglomerular inhibitory circuit (Aungst et al., 2003).

In summary, the glomerulus is the basic unit in the odor processing system in the olfactory bulb. Each odor can activate more than one glomerulus. Within the glomerular layer, excitatory olfactory transmission is modulated in several ways from ON terminals to mitral cells. This complex glomerular network plays a significant role in olfactory processing.

1.2.1.3 External plexiform layer (EPL)

The EPL lies external to the mitral cell layer and deep to the glomerular layer, where the axons of the olfactory nerve (ON) converge onto the dendrites of mitral and tufted (M/T) cells and periglomerular (PG) cells. The EPL of the OB is the second level of synaptic processing. It is mostly composed of excitatory mitral and tufted cell dendrites and GABAergic granule cell dendrites and their synaptic inputs (Huang et al., 2013). Within

the EPL, apical dendrites of inhibitory granule cells form a large number of synapses with M/T cells (Hamilton et al., 2005). The EPL also contains GABAergic interneurons (Duchamp-Viret et al., 1993). These interneurons are excited by M/T cells and provide feedback and lateral inhibition of M/T cells in return (Margrie et al., 2001; Xiong and Chen, 2002). This feedback inhibition plays an important role in olfactory processing (Price and Powell, 1970a, 1970b; Mori et al., 1983).

1.2.1.4 Mitral cell layer (MCL)

The MCL is a thin layer, lying deep to the EPL. Mitral cells (MC) are a key part of the olfactory bulb circuit. MC cells are excitatory in nature and these cells help to connect with other brain regions. These cells receive excitatory input from the axons of ORNs and form synapses in glomeruli. Axons of the mitral cells transfer information to a number of areas in the brain such as the piriform cortex, the entorhinal cortex, and the amygdala through mitral and tufted cell axons forming a tract called the lateral olfactory tract (LOT) (Price and Powell, 1970c; Mori et al., 1983; Shipley and Ennis, 1996).

1.2.1.5 Internal plexiform layer (IPL)

The IPL is a narrow layer lying between the mitral cell layer and granule cell layer. The IPL consists mainly of dendrites from GCs and centrifugal inputs including serotonergic (McLean and Shipley, 1987a), noradrenergic (McLean et al., 1989) and cholinergic (Nickell and Shipley, 1993) inputs. The ET cells send their axons into the IPL, where they form a dense tract that terminates in the granule cell layer (Schoenfeld et al., 1985).

1.2.1.6 Granule cell layer (GCL)

The GCL contains the cell bodies of many interneurons. This layer contains the largest number of cells in the OB. There are over 2 million granule cells (GCs) in each OB of a young rat and over 5 million per bulb in adult rats (Bonthius et al., 1992). GCs are the main cells of the GCL, are very small, axonless and are organized in 3-5 rows in the GCL (Reyher et al., 1991). GCs of the OB are inhibitory in nature (Ribak et al., 1977). These cells form dendrodendritic synapses with mitral cells. In a dendrodendritic synapse, both components release neurotransmitter. So, after excitation of mitral cells, their dendrites release glutamate which in turn excites GC. Excitation of GC leads to release of GABA which inhibits mitral cells. Activation of GC also causes lateral inhibition of other mitral cells. This inhibition helps to improve the signal to noise ratio in odor processing (Price and Powell, 1970b, 1970c).

1.2.1.7 Subependymal layer (SEL)

The SEL lies deep to the ependyma of the lateral ventricle. It is composed of small tightly packed cells that frequently divide. The newly generated cells of the olfactory bulb originate in the SEL (Privat and Leblond, 1972). The cell migration in the mammalian SEL is quite different in neonatal and adult animals. Cells in the SEL are classified into three groups of cells. i) bipolar ii) polygonal and iii) spheroidal cells. Bipolar cells are the major cells of the SEL and they can migrate a very long distance. These cells have an elongated, bipolar shape with two main processes emerging from the opposite poles (Peretto et al., 1999). In rats during their first 3 weeks of life, cells start to migrate towards the OB (Kishi et al., 1990) along a very complicated path of migration known as

the rostral migratory stream (RMS). The RMS tract is connected to the SEL, which is the central part of the OB (Peretto et al., 1999; Sun et al., 2010). Mostly, GABAergic interneurons destined for the olfactory granule cell layers are generated from the subventricular zone (SVZ), whereas periglomerular dopaminergic interneurons are produced from the RMS (Sun et al., 2010). More than 30,000 dividing cells exit from the rodent SVZ for the RMS each day (Lledo et al., 2006). On PND 37, bipolar cells are almost extended from around lateral ventricle to the centre of the OB. A small number of bipolar cells still migrate towards the OB and reach the granular layer. Interestingly, most of the granule cells in the rodent OB are produced in the SEL and this neurogenesis continues up to 31 months after birth (Kishi, 1987; Lledo and Saghatelyan, 2005) .

1.3 Olfactory processing

Odor processing is different from other sensory modalities. Olfaction is the only sense that can reach the cortex without relaying to the thalamus first (Kay and Sherman, 2007). Odor processing involves the conversion of volatile odorants into electrical signals by the use of ORNs. Odorant molecules first bind to the OR on the ORNs, present in the olfactory epithelium. These ORNs transduce the chemical information into an electrical signal and relay to the next olfactory processing structure, the OB (Reed, 1992). There are approximately 1000 odorant receptor types in the mammalian OB and each ORN expresses only one type of odorant receptor (Young and Trask, 2002). Odorant molecules bind to the ORN and activate G-protein-second messenger cascades leading to an influx of sodium (Na^+) & calcium (Ca^+) ions resulting in depolarization of the ORN. As previously described ORN axons synapse with individual glomeruli in the OB glomerular

layer (Breer et al., 1990; Bradley et al., 2005; Kaupp, 2010).

Olfactory processing is quite complex, and several excitatory and inhibitory connections are responsible for carrying olfactory information from the OB to higher order olfactory structures (Adam and Mizrahi, 2010). An odorant is composed of different chemical molecules that bind to specific ORs on the basis of their chemical properties such as functional groups, hydrocarbon structure, and molecular properties. Systematic mapping studies of 365 odorant chemicals activating different ORs reveals a distributed pattern of glomerular activation in the rat olfactory bulb that is related to the chemistry of the odorant stimuli (Krautwurst et al., 1998). This chemotopographical organization suggests that chemical odorants are encoded in different regions of the OB (Jourdan et al., 1980; Johnson and Leon, 2007).

1.4 Neurotransmitters involved in olfactory learning

Olfactory learning is dependent upon synaptic transmission between neurons in the OB. The OB receives central inputs from multiple neuromodulatory regions releasing neurochemicals such as norepinephrine, serotonin, glutamate, dopamine, acetylcholine and GABA (Fletcher and Chen, 2010). In this thesis, I will provide an extended background for three neurochemicals that play vital roles in early olfactory preference learning.

1.4.1 Norepinephrine

The OB receives a dense norepinephrine (NE) projection from the locus coeruleus (LC) The LC projects about 40% of its NE fibers to the OB (Shipley et al., 1985). Within the OB, NE inputs are found in most layers but are densest in the IPL & GCL (McLean et al., 1989). During olfactory preference learning, NE activates the noradrenergic β-adrenorceptors (β-ARs), which serves as an unconditioned stimulus for this learning, while the odor is the conditioned stimulus. Pharmacological blocking of β-ARs in the OB prevents olfactory learning (Sullivan et al., 2000), while pharmacological activation of β-ARs paired with odor produces olfactory learning (Langdon et al., 1997; Sullivan et al., 2000; Yuan et al., 2003; Harley et al., 2006; Lethbridge et al., 2012). These types of bulbar specific manipulations provide the evidence that NE is necessary and sufficient to produce learning (Sullivan et al., 2000). Adrenoreceptors (ARs) are located on several cell types of OB. Both MCs and granule cells express α-ARs including α_1 and α_2 subtypes (Day et al., 1997; Hayar et al., 2001; Nai et al., 2010). β-ARs (both β_1 and β_2) are located in the granule cell, IPL and glomerular layers, but the EPL only expresses β_2-ARs (Woo and Leon, 1995). Antibody localization experiments demonstrated that β_1- ARs are highly expressed on MCs and periglomerular cells with much lower expression on granule cells (Yuan et al., 2003). In 1989, Sullivan et al. showed that blocking of LC-NE input in the OB can inhibit new memory acquisition (Sullivan et al., 1989). Direct stimulation of LC produces odor preference learning (Sullivan et al., 2000).

1.4.2 Serotonin

Serotonin (5-HT) is a neuromodulator in olfactory processing. 5-HT input is present in highest density in the GL (McLean and Shipley, 1987b) but it is also present in the mitral and tufted cell layers of the OB (McLean et al., 1995). 5-HT has a role in olfactory preference memory acquisition in neonatal rats (McLean et al., 1993, 1996). Depletion of 5-HT can impair olfactory preference memory acquisition. This deficit can be overcome by supra levels of β -AR activation in the rat OB (Langdon et al., 1997).

1.4.3 Glutamate

Glutamate plays a vital role in early odor preference learning. In response to the odor, glutamate is released from the ON terminals and binds to MC dendrites within the GL. This glutamate release is thought to mediate the conditioned stimulus (CS) of odorant during odor preference learning in neonate rats (Berkowicz et al., 1994; Yuan et al., 2003). The mitral cells themselves, which provide OB encoding of odors and send odor information out of the OB, also use glutamate as their transmitter. Because of the importance of glutamatergic signaling for encoding in the olfactory bulb, I will provide further details about the receptors for glutamate.

1.5 NMDAR

1.5.1 Structure of the NMDAR

The N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor found on the nerve cell. It consists of a heteromeric complex of four subunits. There are three families of NMDAR subunits named GluN1, GluN2 (A, B, C and D) and GluN3 (A and

B; Cull-Candy et al., 2001). The GluN1 subunit is an essential component of all NMDA receptor complexes. The different four GluN2 subunits (GluN2A-D) are products of separate genes. The functional NMDAR forms a heterotetramer composed of two obligatory GluN1 subunits with two GluN2 or GluN3 subunits. Although GluN3 subunits are expressed throughout the nervous system, their functional roles are not well defined (Cull-Candy et al., 2001).

Each NMDA subunit has a similar structure to other members of the ionotropic glutamate receptor family, with an extracellular N-terminus, intracellular C-terminus, and a re-entrant transmembrane domain. The intracellular C-terminus domain of the subunit interacts with scaffolding proteins and helps in the intracellular modulation such as phosphorylation. L-glutamate is not the only agonist for the NMDAR. Glycine, another amino-acid, is a co-agonist, and both transmitters can bind to the receptor. However, the binding sites for glutamate and glycine are found on different subunits as glycine binds to the GluN1 subunit, while glutamate binds to the GluN2 subunit (Husi et al., 2000; Mayer, 2005). Therefore, both subunit types are required to generate a fully functioning NMDAR. The GluN2B subunit also possesses a binding site for polyamines, regulatory molecules that modulate the physiological functioning of the NMDAR (Erreger et al., 2004).

1.5.2 Roles of the NMDAR in plasticity

The NMDAR acts as a modulator of the synaptic response. The receptors are found in different parts of the brain and are expressed differently at different stages in development. Thus, all types of NMDAR do not act in the same way (Cull-Candy and

Leszkiewicz, 2004). At the resting membrane potential, the NMDAR is inactive. This is due to a voltage-dependent block of the channel pore by magnesium (Mg^{2+}) ions.

To unblock the channel, the postsynaptic cell must be depolarized. When glutamate is bound to the receptor and the postsynaptic cell is depolarized such that the Mg^{2+} block is removed from the channel, cations flow through (Mayer et al., 1984). The NMDAR is permeable to sodium, potassium, and importantly calcium ions. This NMDAR activation leads to calcium influx into the post-synaptic cells resulting in the activation of a number of signaling cascades. In this way, the NMDAR plays a well-defined role in cellular activation mediated synaptic plasticity (Bear and Malenka, 1994; Malenka and Bear, 2004).

1.5.3 NMDA downregulation and unlearning

Lethbridge et.al.(2012) reported that GluN1 is downregulated 3 h after odor preference training and suggested this downregulation may assist memory stability (Lethbridge et al., 2012). More recently, data published by Mukherjee et al. (2014) have shown that retraining at 3 h interferes with the expression of odor preference induced by initial exposure. This learning impairment is correlated with NMDAR downregulation at 3 h after first odor preference training. Since NMDAR blockade restores normal learning at 3 h, the study reveals a strong associative relationship between NMDAR downregulation and unlearning (Mukherjee et al., 2014). NMDAR blockade also prevents normal odor preference learning in the rat pup (Lethbridge et al., 2012). These results provide direct support for a critical role of NMDARs in early odor preference learning.

1.6 AMPAR

1.6.1 Structure of the AMPAR

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) is an ionotropic transmembrane receptor in the central nervous system (CNS). The AMPAR consists of four types of subunits, GluA1, GluA2, GluA3, and GluA4. Like all the ionotropic glutamate receptor subunits, GluA1 subunits have an extracellular N-terminus and an intracellular C-terminus (Gouaux, 2004). Most AMPAR are heterotetrameric consisting of symmetric two dimers of GluA2 and either GluA1, GluA3 or GluA4. The subunit composition of the AMPAR is also important for the way this receptor is modulated. GluA2 is a critical subunit in determining mammalian AMPAR function. This subunit determines many of the major biophysical properties of the native receptor, including, receptor kinetics, single-channel conductance, calcium permeability. Most AMPA receptor channels are impermeable to calcium, a function controlled by the GluA2 subunit. GluA2-containing AMPARs are thought to be the most abundant AMPAR in the CNS. During early postnatal development, expression of GluA2 is low compared with that of GluA1, but it increases rapidly during the first postnatal week (Traynelis et al., 2010).

Though GluA2-lacking AMPARs are calcium permeable, such receptors play a major role in neonatal synaptic function. In addition, throughout the brain, GABAergic interneurons, which represent about 10% of the total cell population, exhibit low levels of GluA2 subunit expression, and some of these neurons express a significant proportion of GluA2-lacking calcium-permeable AMPARs at all developmental stages under certain physiological or pathological conditions (Isaac et al., 2007; Bowie, 2012).

1.6.2 Roles of the AMPAR in plasticity

AMPA receptors are responsible for glutamate mediated fast excitatory synaptic transmission throughout the CNS. Their modulation plays a vital role in synaptic plasticity, as mediators in long-term memory (Malenka and Bear, 2004). Increasing the post-synaptic response to a stimulus is achieved either by increasing the number of AMPA receptors at the post-synaptic surface or by increasing the single channel conductance of the receptors expressed, previously shown to be the basis of LTP. Physiological studies have shown that synaptic plasticity may be increased by AMPAR insertion rather than a modification of existing receptors (Nayak et al., 1998; Lu et al., 2001; Malinow and Malenka, 2002). Interestingly, olfactory preference training leads to increase membranous AMPA receptors expression in the GL at the time of the 24 h olfactory preference memory. This increase is no longer seen 48 h post-training and correlates with decreased memory retention at 48 h (Cui et al., 2011).

1.7 Models for the odor preference memory

The early odor preference learning is a classical conditioning model (Wilson and Sullivan, 1994; Sullivan, 2003). There are several types of stimuli that can be used as an unconditioned stimulus to induce conditioned responses to a novel odor in neonates such as stroking or tactile stimulation (Sullivan and Leon, 1986; Sullivan and Hall, 1988; Weldon et al., 1991; Moore and Power, 1992; McLean et al., 1993), tail pinch (Sullivan et al., 1986), milk presentation (Johanson and Hall, 1982; Johanson et al., 1984; Sullivan and Hall, 1988) and mild foot shock (Camp and Rudy, 1988; Roth and Sullivan, 2001;

Sullivan, 2003; Moriceau et al., 2006). The aversive stimuli, tail pinch or mild foot shock (0.5 mA), paired with an odor can only induce preference memory in the first week of life. In the second and third postnatal weeks, mild foot shock paired with odor induces aversive memory in pups (Camp and Rudy, 1988; Sullivan et al., 2000; Moriceau et al., 2006).

Leon and his colleagues (Leon et al., 1977; Coopersmith and Leon, 1984) first showed that peppermint odor exposure from PND 1-19 (3 h/day) could induce preference memory for peppermint at PND 20. Later, another experiment by Sullivan and colleagues revealed that a clear odor preference could also be observed on PND 19 with odor + stroke training for 10 min/day at PND 1-18 (Sullivan and Leon, 1986). However, pups trained- with an odor and intense tactile stimulation after the first postnatal week (after the first 10 days) do not develop a preference memory for the trained odor. These results suggest that PND 1-10 is a sensitive period for the development of early odor preference memory (Woo and Leon, 1987).

1.8 Neonatal odor preference learning model

Neonatal odor preference learning is an excellent associative learning model. During early life, the CNS is not fully developed. However, pup behaviour is governed by maternal interactions and odor is their primary cue for guidance. As a result, pups use a different circuitry than adult rats to form new memories (Sullivan and Wilson, 2003; Moriceau and Sullivan, 2004). In the model, as described, tactile stimulation (stroking with a paint brush) is paired with a novel odor to form new memories in a rat pup. This is important learning for pup survival during this early critical developmental period

(Sullivan and Leon, 1986; Wilson and Sullivan, 1994). The locus coeruleus (LC) and the OB play the critical roles in odor learning in neonatal rats during this period when other parts of the brain are less developed. A single 10 min training session involving stroking paired with novel odor releases norepinephrine (NE) in the olfactory bulb (Rangel and Leon, 1995). The LC is uniquely sensitive to stroking in this period (Nakamura et al., 1987). The release of NE and the resulting β-AR activation from this single pairing event is sufficient to produce 24 h odor preference learning in the neonatal rat (Sullivan et al., 1989, 1991; Harley et al., 2006). This suggests that when pups are completely dependent on the dam for survival, they are biased to achieve associations with stimuli associated with her care.

1.9 Role of protein transcription and translation in learning and memory

On the basis of their temporal features, memories have been characterized in phases including short-term memory (STM), intermediate-term memory (ITM) and long-term memory (LTM). These phases of memory are also differentiated by their dependence on transcription and translation. In 2011, Grimes et al. characterized these three types of memory in the early odor preference learning model. They showed that ITM (5 h memory) is disrupted by a protein translational inhibitor (anisomycin) infused into the OB but not by a transcription inhibitor (actinomycin; Grimes et al., 2011). Infusion of either the translation or transcription inhibitor prevents LTM (24 h memory), whereas, neither inhibitor affects STM (3 h memory). This study implies that protein synthesis (translation and transcription) is critical for long-term odor preference memory but not STM (Grimes et al., 2011, 2012) consistent with other mammalian learning models.

1.10 Epigenetic regulation in memory formation and maintenance

It is well known that even long-term memory can have multiple durations all of which involve translation and transcription. In recent years, several epigenetic regulation studies have suggested that longer duration memory depends on such regulation (Guan et al., 2002). Epigenetic mechanisms such as chemical modifications of DNA and histones (acetylation and deacetylation) appear to play vital roles in memory formation and maintenance over longer time frames (Levenson and Sweatt, 2005; Zovkic et al., 2013).

1.10.1 Histone

1.10.1.1 Structure and function of histone

Histones are alkaline proteins found in eukaryotic cell nuclei. They associate with negatively charged DNA and form a nucleosome structure. The nucleosome core consists of octameric protein bound by 147 base pairs of DNA. In the nucleosome core, there exist four major families of histone including H2A, H2B, H3, H4 and two linker histones including H1 and H5. Two of each of the core histones assemble to form one octameric nucleosome core. The linker histones are associated with the DNA as well as the core nucleosome histones and play an important role in the compaction of DNA within the nucleosome unit (Smith, 1991; Ramakrishnan, 1997).

The four core histones are similar in structure and highly conserved. All histones are dynamically modified in a highly regulated manner to modulate chromatin assembly (Shechter et al., 2007). Modification of histones includes acetylation, phosphorylation, ubiquitination and methylation (Iizuka and Smith, 2003). The biological significance of these modifications is not well understood, but the general hypothesis that they play a role

in determining the state of gene activity has been confirmed (Luger and Collins, 2001; Mariño-Ramírez et al., 2005).

1.10.1.2 Role of histone modifications in learning and memory

Post-translational modification of histones plays an important role in transcription activity (Grunstein, 1997). One of these modifications is regulated by two histone-related enzymes including histone acetyltransferase (HAT) and histone deacetylase (HDAC; Hebbes et al., 1988). During protein synthesis, DNA needs to be in a relaxed condition. For DNA relaxation, negatively charged acetyl groups are added to histone proteins by HATs resulting in changing their affinity for DNA. This relaxed conformation of chromatin makes the DNA accessible for gene transcription (Brownell and Allis, 1996). In contrast, HDAC plays the role of reversing this process, where acetyl groups are removed from histone proteins allowing normal nucleosome structure that is transcriptionally inactive (de Ruijter et al., 2003; Vecsey et al., 2007). Thus HDACs normally work as transcription repressors. Several pharmacological studies have indicated that HDACs are critical negative regulators of long-term memory formation (Qiu, 2009).

CREB binding protein (CBP) is a co-activator with HAT activity. Histone acetylation is a potential enhancer for CBP-dependent CREB induced gene transcription to recruit new genes for transcription mechanisms (Korzus et al., 2004). CBP mutant mice exhibit significantly impaired specific forms of long-term memory including long-term memory for contextual fear. CBP mutant mice also exhibit significantly impaired hippocampal-dependent long-term memory for object recognition. Together, these results indicate that HDAC inhibition modulates memory formation via CBP and different brain regions can

utilize chromatin-modifying enzymes (HAT/HDAC) to regulate learning and memory (Wood et al., 2006; Stefanko et al., 2009; Haettig et al., 2011). In contrast to the genetic studies for examining the role of CBP in memory, the majority of studies examine HDAC involvement in memory by using pharmacological approaches. Several studies suggest that HDAC2, but not HDAC1 ,is the key HDAC in regulating memory formation (Guan et al., 2009). Recently, a study showed that HDAC3 is also a critical negative regulator of memory formation (McQuown et al., 2011). However, the specific HDAC subtype involvement in memory formation is still not clear (Haettig et al., 2011).

It is now accepted that histone acetylation, achieved by inhibiting deacetylation via HDAC inhibitors, is a major molecular mechanism in the regulation of transcription underlying memory extension (Fischer et al., 2007; Haettig et al., 2011). HDAC inhibition in long-term memory was first used in a contextual fear conditioning study. In that study, contextual fear conditioning memory and LTP were both induced by increasing acetylation of H3 in the hippocampus of fear conditioned rats with trichostatin-A (TSA), an HDAC inhibitor (Levenson et al., 2004). A study by Vecsey et al. (2007) showed that TSA modulates CBP - CREB interactions and histone acetylation required for contextual fear memory consolidation. They suggested that CBP - CREB interaction and histone acetylation provide regulatory mechanisms for enhancing memory and synaptic plasticity using HDAC inhibition (Korzus et al., 2004; Vecsey et al., 2007). Recent findings show that intrabulbar infusion of TSA facilitates aversive olfactory learning in young rats (Wang et al., 2013). However, all of these studies are related to aversive learning. Surprisingly, there is no HDAC inhibition mediated prolonged single trial preference memory model reported in the literature. In recent years, researchers have tried to use

HDAC inhibitors as a successful novel therapeutic strategy for the improvement of memory deficits in several neurodegenerative animal models including those for Alzheimer's and Huntington's disease (Kilgore et al., 2010; Giralt et al., 2012).

1.10.1.3 Histone deacetylase (HDAC)

HDACs are a class of enzymes that remove acetyl group from lysine amino acids on a histone to promote gene transcription. HDACs are classified in four classes depending on the sequence homology of the enzyme and are categorized as Class I, II, III and IV (Leipe and Landsman, 1997).

Among these four classes, the Class IIs have two sub families; Class IIA and IIB.

Members of HDAC family:

Class I- HDAC 1, 2, 3 and 8

Class IIA- HDAC 4, 5, 7and 9,

Class IIB – HDAC 6 and 10

Class III- sirtuins in mammals, SIRT1, 2, 3, 4, 5, 6 and 7

Class IV- HDAC 11

Within the Class I HDACs, HDAC 1, 2 and 8 are found in the nucleus .Whereas, HDAC3 is found in both the nucleus and the cytoplasm. Class II HDACs are able to shuttle in and out of the nucleus and are involved in a variety of biological processes. Subcellular distribution of class III is not well characterized. Class IV, HDAC 11 is a cytoplasmic enzyme (Leipe and Landsman, 1997).

Trichostatin-A (TSA), HDAC inhibitor:

TSA is a natural compound that serves as an inhibitor of the class I and II mammalian HDAC families of enzymes, but not the class III HDACs. The first two classes of HDAC are considered ‘classical’ HDACs whose activities are inhibited by TSA. During HDAC inhibition, TSA occupies an active site on the target HDAC enzyme and acts as inhibitor by chelating zinc (Zn 2+) ions from the active site (Finnin et al., 1999; Monneret, 2005; Dokmanovic et al., 2007).

1.11 Objectives and hypothesis

The main purpose of this thesis is to establish a prolonged odor preference memory model. In this study, I test the role of histone acetylation in odor preference memory in week-old rat pups following a single training trial that normally only induces 24 h memory. First, it is hypothesized that TSA will promote a longer term odor preference memory. Second, as previous studies from our laboratory (Cui et al., 2011) showed that AMPA receptor (GluA1) membrane localization was increased at 24 h and was no longer observed 48 h after training when odor preference is no longer expressed behaviorally, I hypothesized that HDAC inhibition would maintain enhanced GluA1 receptor levels longer in a prolonged odor preference memory model. Lastly, I looked at the specificity of prolonged odor preference memory for the paired odor and the duration of HDAC inhibition following a single infusion. I also examined the role of HDAC inhibition in NMDA mediated unlearning.

CHAPTER 2- METHODS

2.1 Animals:

Sprague Dawley (Charles River, Saint-Constant, Quebec, Canada) rat pups of both sexes were used in this study. The day of birth was considered PND 0. Litters were culled to 12 rat pups on PND 1. Not more than one animal of each sex, per litter, was assigned to each training condition. Animals were housed in temperature-controlled rooms (20-25^{o}C) on reverse 12 h light/dark cycles. All experimental procedures were approved by the Institutional Animal Care Committee at Memorial University of Newfoundland following the guidelines set by the Canadian Council on Animal Care and the protocol number is 14-01-M.

2.2 Cannula surgery:

Two cannulae were anchored in dental acrylic (Lang Dental, Wheeling, IL, USA) such that they were separated laterally by approximately 4 mm and extended beyond the acrylic approximately 0.5-1mm. When the dental acrylic had hardened, excess acrylic was trimmed off to make the cannulae assembly as small as practical. The cannulae were sonicated to remove excess acrylic particles inside the cannulae. Insect pins (size 00, diameter 0.3 mm, Ento Sphinx, Pardubice, Černá za Bory, Czech Republic, cat. No. 01.20) were placed inside the cannulae to prevent blocking.

On PND 5, rat pups were anaesthetized by hypothermia and placed in a stereotaxic apparatus. The skull was exposed and two small holes were drilled over the central region

of each olfactory bulb. The cannulae were implanted into the olfactory bulb and cemented to the skull. The skin was sutured together and pups were allowed to recover from anesthesia on warm bedding before being returned to the dam.

2.3 Drug preparation:

TSA (Cedarlane, Burlington, Ontario, Canada, cat. No. T-1052) was dissolved in 100% dimethyl sulfoxide (DMSO, EMD chemicals Inc., Darmstadt, Hesse, Germany, cat. no. CAMX1456-6) to prepare a stock solution (stock concentration 1µg/ µl) and aliquoted as 10 µl in each vial to store at -20^{o}C. To make the experimental concentration, 10 µl TSA was diluted with 190 µl of 10% DMSO to make 200 µl total volume (working concentration 0.05 µg/µl) as described previously (Wang et al. 2013).

2.4 Drug infusion:

Infusion cannulae were made from 30 gauge stainless steel tubing cut to a length of approximately 7 mm and inserted into PE-20 polypropylene tubing (inner diameter 0.38 mm , outer diameter 1.09 mm, Becton Dickinson, Sparks, MD, USA, cat. No. 427406). For bilateral OB infusion, the end of the PE-20 tubing was attached over the needle of a 10 µl micro-syringe (cemented needle 26s gauge, needle length 2 inch, Hamilton Company, Reno, NV, USA, model no. 701 N). On PND 6, 20 min before training, pups received intrabulbar infusion of 1.0 µl of 0.05 µg TSA into each olfactory bulb via the cannula implanted the previous day. Hamilton syringes and a multi-syringe pump (Harvard Apparatus, Holliston, MA, USA, model no. Pump11 Elite) were used to infuse 1 µl of TSA into each olfactory bulb over a 3 min period.

2.5 Odor preference training and testing procedure:

2.5.1 Training:

A single 10 min training session was performed on PND 6 rat pups in temperature controlled (28^{o}C) behaviour rooms. After the drug infusion, pups were placed on peppermint-scented bedding (most experiments) or orange-scented bedding (one experiment, see Result section 3.6, Fig.7) for 10 min and stroked with a paint brush for 30 sec every other 30 sec. Pups in the non-learning condition were placed on the peppermint-scented bedding for 10 min without stroking. Peppermint-scented bedding was prepared by adding 0.3 ml of peppermint extract (G. E. Barbour Inc., Sussex, NB, Canada) to 500 ml of regular unscented woodchip bedding and covered for 10 min. Orange-scented bedding was prepared in a similar way by adding 0.3 ml of orange oil (natural, cold compressed, California origin, Sigma, St. Louis, Missouri, USA, cat. no.W282510) to 500 ml of regular unscented woodchip bedding. Peppermint- or orange-scented bedding was then left uncovered in a fume hood for 10 min allowing any solvent to evaporate. Pups were returned to the dam immediately after training until sacrifice or further testing. For the re-training behaviour experiment, pups were re-trained at 3 h after the first training. Pups were exposed to peppermint-scented bedding while being stroked using the same procedure as in the first training. Pups were returned to the dam after re-training.

2.5.2 Testing:

During testing, a two odor choice test was carried out in a stainless steel test box placed over two training boxes. For the majority of tests, one box contained peppermint-scented bedding, and the other contained normal, unscented bedding. Testing boxes were separated by a 2 cm neutral zone. To begin testing, each rat pup, one at a time, was removed from the dam and transferred to temporary holding cage with no bedding and kept in the testing room where the dam and other pups are being caged to prevent odor contamination. To start the testing, the pup was placed in the neutral zone of the test box. The amount of time the pup spent on either peppermint-scented bedding or normal bedding was recorded during each of five 1 min trials. The average time spent over peppermint-scented bedding or normal bedding was calculated for each pup. Pup was given 30 sec resting time between each of five 1 min trials. During this 30 sec resting time, the pup was returned to the holding cage. In two experiments, pups were tested with orange-scented bedding vs. unscented bedding and the percentage of time the pup spent over the orange-scented bedding was calculated (see Result sections 3.5, 3.6, Fig. 6, 7).

2.6 Experiment 1

2.6.1 Behavioural procedures of immunohistochemistry (IHC)

For histone acetylation (AcH3 and AcH4) expression at 30 min and 2 h, intra-animal controls were used. To accomplish this, TSA was infused into one bulb and vehicle was infused into the contralateral bulb. After the drug infusion, pups were exposed to

peppermint-scented bedding for 10 min or stroked with a paint brush (see Results section 3.1, Fig.1). Pups were sacrificed by perfusion followed by immunohistochemistry as described in Section 2.6.2.

In a second experiment, AMPA (GluA1) receptor expression was examined by immunohistochemistry in the olfactory bulb 5 days (5D) after odor-stroke training. Pups were infused with a drug in a similar manner as the AcH3 IHC experiment described in the preceding paragraph. After infusion, pups were exposed to peppermint paired with 10 min of stroking using a paint brush (see Result section 3.4, Fig.4, 5). Pups were perfused 5D after training and brains processed for immunohistochemistry as described in Section 2.6. 2.

2.6.2 Perfusion and IHC procedures

At 30 min, 2 h (AcH3 and AcH4) and 5D (GluA1) after training, animals were anaesthetized by sodium pentobarbital (80 mg/kg, Bimeda-MTC Animal Health Inc., Cambridge, Ontario, Canada, cat.no. N05CA01) and perfused transcardially with ice-cold 0.9% saline solution (~1 min) followed by ice-cold fixative: 4% para formaldehyde (Fisher Scientific, Hampton, New Hampshire, USA, cat. no.T353) in 0.1M phosphate buffer, pH 7.4. Brains were removed from the skull and post-fixed for 1 h in the same fixative solution, after which they were immersed in 20% sucrose solution overnight at 4^0C.

The next day, brains were flash-frozen and 30 μm coronal sections of the entire bulb were cut in a cryostat (Thermoscientific, Waltham, MA, USA, model no. Microm HM550) at -15^0C. Sections were directly mounted onto slides. The primary antibodies rabbit

AcH3(1/5000,Cell Signaling, Danvers, MA, USA, cat.no.9649), rabbit AcH4(1/10000, Cell Signaling, Danvers, MA, USA, cat.no.2594), and rabbit GluA1 (1/10000,Abcam,Cambridge, MA, USA, cat.no. ab109450) were diluted in phosphate buffered saline with 0.2% TritonX-100, 0.002% sodium azide and 2% normal goat serum and applied to sections overnight at 4^0C. The next day, sections were washed in PBS for 3x5 min and incubated in a biotinylated anti-rabbit secondary antibody (Vectastain Elite, Vector Laboratories Inc., Burlington, Ontario, Canada, cat. no. PK-6101) dissolved in phosphate buffered saline (PBS) with 0.2% TritonX-100 for 1 h. Then sections were washed in PBS for 3x5 min and avidin /biotinylated enzyme (A+B) solution (Vectastain Elite, Vector Laboratories Inc., Burlington, Ontario, Canada, cat. no. PK-6101) was added followed by incubation for 1 h. Next, sections were incubated in 0.05% diaminobenzidine (DAB) with 0.01% hydrogen peroxide (H_2O_2) for 3 min and washed in PBS for 3x5 min. Then slides were rinsed with distilled water for 10 sec. Afterwards, sections were dehydrated and cover-slipped with Permount® (Fisher Scientific, Hampton, New Hampshire, USA, cat. no. SP15). Olfactory bulb sections (vehicle- treated side and TSA-treated side) from each pup were processed on same slide at identical times for incubation in the antibodies and development in DAB solution. Thus, this drug infusion and processing methods provided intra-animal control for IHC.

2.6.3 Image analysis for GluA1 IHC

Images of sections were captured with a CCD camera (Leica Microsystems Ltd., Heerbrugg, Sankt Gallen, Switzerland, model no. Leica DFC 495) connected to the Leitz microscope (Leitz, Stuttgart, Baden-Württemberg, Germany, model no. Diaplan) at 4x

magnification. Leica Application Suite software (Leica Microsystems Ltd., Heerbrugg, Sankt Gallen, Switzerland, version 4.2.0) was used to process and store the images. The light intensity of the microscope was kept at the same level for all the sections from all animals analyzed. The intensity of GluA1 staining was analyzed using Image J analysis software (National Institutes of Health, Bethesda, Maryland, USA, version 1.46p). Regions analyzed included the dorsolateral and dorsomedial quadrants of the GL known to be responsive to peppermint (Johnson and Leon, 2007) and the dorsolateral and dorsomedial quadrants of EPL for each section in the olfactory bulb. To analyze the sections, the optical density (OD) of the olfactory nerve layer was used as a background OD for the section. This was accomplished by drawing four 100 μm diameter circles on the olfactory nerve layer in each section and averaged to obtain the OD reading. To measure the OD of the glomeruli, 8 glomeruli were marked in each quadrant and the OD averaged for that quadrant in each section. The external plexiform layer was analyzed similarly by drawing 8 (100 μm diameter) circles in each quadrant and averaging the OD. Measurements of OD were taken from 4 rostral to caudal levels of the glomeruli and external plexiform layers from each pup. The relative OD of the region of interest (ROI) was obtained by using the following formula: (OD of background − OD of ROI)/OD of background.

2.7 Experiment 2:

2.7.1 Experimental Groups for Western Blotting:

Rat pups were divided into three groups: Saline+Odor, Saline+Odor+Stroking and TSA

+Odor +Stroking. Each group was further subdivided into four time points (3, 24, 48 h and 5D) after training.

2.7.2 Tissue collection:

Samples were collected at four different time points after odor conditioning: 3, 24, 48 h and 5D). After decapitation, olfactory bulbs were quickly removed and frozen on dry ice. Tissue was stored in lysis tubes (Micro tube 0.5ml, SARSTEDT AG & Co., Nümbrecht, North Rhine-Westphalia, Germany) containing beads (1.4 mm Zirconium oxide beads, Precellys ®24) at -80^{0}C until processing.

2.7.3 Synaptic and extra-synaptic protein extraction protocol:

Tissues were homogenized in 100 µl sucrose buffer (see composition in Appendix 1) at 5500 rpm for 20 sec in an homogenizator (Precellys® 24). The solution was transferred into 1.5 ml centrifuge tubes. The homogenate was centrifuged at 1000xg at 4^{0}C for 15 min to remove nuclei and incompletely homogenized material (P1). The supernatant (S1) was collected into another 1.5 ml tube and centrifuged at 10,000xg at 4^{0}C for 20 min to obtain the membrane fraction (P2) and to remove the cytosolic fraction (S2). The pellet (P2) was resuspended in 80 µl 1X STE [sodium dodecyl sulfate (SDS) tris-ethylenediaminetetraacetic acid (EDTA), see composition in Appendix 1] buffer. The samples were sonicated and then heated to 90^{0}C for 3 min.

2.7.4 Protein concentration determination:

Bovine serum albumin (BSA, 2.0 mg/ml, Pierce BCA Protein Assay kit,

Thermoscientific, Waltham, MA, USA, cat.no. 23225) was used as the standard for protein determination in 1/10 dilution. 25 µl samples and 200 µl bicinchoninic acid (BCA) were loaded into each well of a 96 well plate. Plates were incubated at 37^0 C for 30 min. Next, plates were read using a Microplate Reader (BMG Technologies Inc., Clearwater, FL, USA, model no. Polar star Optima) at 562 nm. Relative optical densities were plotted to a standard curve [standards of BSA and water were prepared in a serial dilution (0 µg, 5 µg, 10 µg, 15 µg, 20 µg, 25 µg, 30 µg, and 40 µg)] and calculated to determine the volume required to load 50 µg of protein per sample into the wells of polyacrylamide gels used for electrophoresis (see 2.7.5).

2.7.5 Western Blotting:

Samples were prepared by aliquoting 50 µg of protein lysate mixed with 5x sample buffer (see composition in Appendix 2) and water (to adjust total volume to 25 µl). Samples were mixed and boiled for 5 min at 100^0 C. Then samples were loaded into each well of a 10-well, 10% polyacrylamide gel with 2.5 µl of protein ladder (Page Ruler Plus, Thermoscientific, Waltham, MA, USA, cat. no. 26619). A Biorad (Bio-Rad, Hercules, CA, USA) electrophoresis power supply was used to run gels at 60 mA for 1.5 h until the loading dye band reached the bottom of the gels.

Gels were separated from the glass plates and placed on 3 mm chromatographic paper (Whatman Plc., Maidstone, Kent, England). Nitrocellulose membrane (Millipore, Billerica, MA, USA) was placed on top of the gels followed by a piece of chromatographic paper. Chromatographic paper, gel and nitrocellulose membrane were

sandwiched by sponges (pre -soaked in transfer buffer) and placed in transfer cassette grids, which were then placed in a transfer apparatus (Bio-Rad, Hercules, CA, USA) filled with transfer buffer. Blots were transferred at 100 volts for 60 min at 4^0 C.

Nitrocellulose membrane was removed from the cassette and washed in tris-buffered saline with 0.1% tween (TBST, see composition in Appendix 2) for 3x5 min. Blots were blocked in 5% non-fat dry milk + TBST for 1 h at room temperature. After washing, blots were incubated in primary antibody (in 5 % non-fat dry milk + TBST) overnight at 4^0 C on a continuous vertical shaker, so that the solution could move over the blot thoroughly. The next morning, blots were washed in TBST (3x10 min) and incubated in secondary antibody (goat anti-rabbit conjugated with horseradish peroxide (Thermoscientific, Waltham, MA, USA, cat. no. 31466) with 5% non-fat dry milk +TBST, for 1.5 h at room temperature and then washed in TBST (3x10 min).

Blots were immersed in Enhanced Chemiluminescence (ECL, Thermoscientific, Waltham, MA, USA, cat. no. 34080) substrate in a 1:1 ratio (super signal west pico stable peroxide solution : super signal west pico luminol/enhancer solution) for 2 min at room temperature. Blots were then placed on a plastic sheet and excess ECL was removed by wiping with a Kimwipe®. Blots were placed in a film box and exposed for varying length of time to film (18x24 cm, Kodak Clinic Select Green) in a dark room using a mini medical 90 developer (AFP Imaging Corp., Elmsford, NY, USA).

For examining AMPA receptor (GluA1) expression, rabbit polyclonal GluA1 (1/5000,

Abcam, Cambridge, MA, USA, cat.no. ab109450) was used. In the experiments looking for GluN1 expression at 3h after odor preference training, rabbit monoclonal GluN1 (1/2000, Cell Signaling, Danvers, MA, USA, cat.no. 5704) and β-Actin (purified rabbit anti-β-actin, 1/5000, Cell Signaling, Danvers, MA, USA, cat. no. 4967) as a loading control was used to check GluA1 and GluN1 expression .

2.8 Statistics:

In behavioural experiments, one-way ANOVA with a Tukey's post hoc test was used to make comparisons among different groups. In western blotting experiments, GluA1 expression was analyzed by two-way ANOVA followed by Tukey's post hoc comparisons with both time and groups as factors, while GluN1 expression at 3 h after training was accessed by unpaired t-test. During image analysis for GluA1 IHC, I used the paired t-test to compare saline-treated OB vs. TSA-treated OB obtained from the same animals. A mixed factorial designed ANOVA was used to compare the groups (TSA-treated vs. saline-treated) and a between-subjects factor and conditions (time spent over orange at 3d vs. time spent over peppermint at 5D), as a within-subjects factor. An unpaired two-tailed t-test was performed to evaluate the memory specificity to the paired odor between two groups and also in 3 h retraining behavioural experiments. Differences between groups were considered significant when p values were <0.05.

CHAPTER 3 – RESULTS

3.1 Qualitative immunohistochemical observation of one or more subtypes of histone acetylation

HDAC inhibition was examined at 30 min and 2 h following odor training using IHC. TSA was infused in one OB and vehicle (saline) in the other OB. Animals were trained in the presence of odor + stroke (O/S) or odor only (O/O). There were no detectable differences in histone acetylation observed between the two OBs of the odor+stroke animal (TSA-treated vs. vehicle-treated side, Fig.1A). However, an increased level of acetylation of H3 and H4 was observed in TSA-treated OB compared with vehicle-treated OB in odor only trained pups at both the 30 min and 2 h time points (Fig. 1B). These qualitative results support the basic concept that HDAC inhibition can be involved in one or more subtypes of histone acetylation in the OB. These results also confirmed that the TSA was infused in the right place of the OB. Fig.1A also suggests that stroking itself can lead to acetylation of histone.

3.2 HDAC inhibition extends odor preference memory

To identify the role of HDAC inhibition in prolonging memory, I designed an odor preference memory experiment testing at different memory durations 2 days (2D), 4 days (4D), 5 days (5D), and 9 days (9D). Our behavioral data revealed a robust memory in the TSA-treated groups compared with the control groups. At 2D following training on PND 6, animals that received bilateral intrabulbar infusion of TSA combined with odor + stroke training showed significantly greater preference for peppermint odor compared to

the control group (Fig. 2A, $p < 0.001$). To investigate how long the increased level of preference memory could last, preference testing was carried out on individual rat pups at 4D, 5D and 9D post-training (Fig. 2B-D). Remarkably, the preference memory was observed in the TSA-treated group up to 9D after the 10 min training session on PND 6 (4D: $p < 0.001$; 5D: $p < 0.001$; 9D: $p < 0.001$).

3.3 GluA1 expression is increased at 48 h after training in a TSA-treated odor preference memory model

The effect of TSA-induced prolonged odor preference memory was explored to determine if it could be correlated with prolonged GluA1 expression in the olfactory bulb. I used a western blot technique at three different time points (24 h, 48 h, and 5D). Animals received either saline paired with an odor, saline with odor+stroke or TSA paired with odor + stroke. At 24 h following training, the expression of the GluA1 subunit (shown as relative optical density) from synaptic and extra-synaptic membrane fractions in the TSA+O/S and Sal+O/S groups did not differ significantly (Fig. 3A, $p > 0.05$).

At 48 h following training, a significant increase of GluA1 expression was observed in TSA + O/S group (Fig. 3B, $p < 0.05$) compared to the Sal + O/S group. GluA1 expression in TSA + O/S group did not show any significant difference with Sal+ O/S group (Fig. 3C, $p > 0.05$) 5D following training.

3.4 Immunohistochemical (IHC) expression of GluA1 in the glomerular layer of the olfactory bulbs at 5D after training

To further explore GluA1 expression at 5D after training, IHC was performed. In this study an intra-animal control was used, where TSA was infused into one OB and saline

into the contralateral side after which the pup was trained with odor +stroke pairing. Quantitative analysis of GluA1 expression revealed a significantly increased level of the GluA1 label within the dorsolateral and dorsomedial quadrants of the glomerular layer (Fig. 5B-C, dorsolateral: $p < 0.05$; dorsomedial: $p < 0.05$) in the TSA infused side compared with vehicle infused side (Fig. 4B-C). There was no significant difference of GluA1 expression observed in the external plexiform cell layer (EPL) of the dorsolateral and dorsomedial quadrants (Fig. 5D-E, dorsolateral: $p > 0.05$; dorsomedial: $p > 0.05$) in the TSA-treated vs. vehicle-treated side (Fig. 4B-C). This is consistent with a strengthening of input to odor encoding glomeruli through increased AMPA receptor insertion that is still observable 5D after training.

3.5 TSA induced odor preference memory is specific to paired odor

In order to identify whether TSA induced odor preference is specific to the paired odor or not, I designed a second behavioural experiment. In this study, I trained the TSA infused rat pups in the presence of peppermint + stroking and tested them in the presence of either peppermint or orange odor 5D after training. Pups have the ability to show preference to the normally aversive orange odor if they are trained in the presence of orange odor (Grimes et al., 2015). My data showed the time spent over peppermint (conditioned odor) was significantly greater (Fig. 6, $p < 0.0001$) compared to the time spent over orange (non-conditioned odor). This suggests TSA induced odor preference memory is specific to the paired odor.

3.6 How long does TSA remain effective?

I designed another behavioural experiment to determine if TSA remains effective in the OB the day after TSA infusion. On the first day of training on PND 6, rat pups were trained with peppermint + stroke in the presence of TSA or saline and the next day the same pups were re-trained with Orange + stroke. If the TSA remained active in the olfactory bulb at the time of the orange odor training, one might expect the pups trained to orange odor would remember that odor several days later. Odor preference testing was performed at PND 10 with orange and PND 11 with peppermint. Normally, orange is aversive to rat pups (Grimes et al., 2015) but it can act in a similar way as peppermint in 24 h odor preference learning with O/S training. The TSA-treated pups spent significantly more time over the peppermint than the orange odor (Fig. 7, $p < 0.0001$). The vehicle-treated groups did not show any preference for peppermint or orange. This result suggests that TSA was no longer effective 24 h after infusion, since pups trained to orange odor the day after they were trained to peppermint odor and TSA treatment did not show memory enhancement.

3.7 TSA can abolish unlearning in retrained pups at 3 h

In this study, animals were trained in two trials separated by a 3 h interval. The animal received either saline or TSA paired with peppermint odor +stroking. During the first training session, pups were trained with peppermint odor +stroking. Those groups were retrained at 3 h using the same odor + stroking training procedures. Interestingly, TSA-treated pups which were trained two times in peppermint bedding with a 3 h interval showed a higher preference learning for the peppermint 21 h after a second session of

training compared with the vehicle-treated O/S group (Fig. 8, $p< 0.01$). This result suggests that retraining at 3 h interferes with the expression of the odor preference induced by the initial exposure in the vehicle-treated odor-stroke group as shown previously by (Mukherjee et al., 2014) but not in the TSA-treated groups. In other words, TSA induced odor preference training impairs unlearning at 21 h after a second session of odor preference training.

3.8 GluN1 down-regulation occurs in vehicle-treated groups at 3 h but not in TSA - treated group

Lethbridge et al. (2012) showed that the GluN1 subunit was down-regulated 3 h after early odor preference training in the OB. In this study, I tested whether TSA could block G1uN1 down-regulation at 3h after odor preference training. I used a western blot technique at 3 h after training to examine this question. Animals received either saline paired with an odor, saline with odor + stroking or TSA paired with odor + stroking. At 3 h following training on PND 6, the expression of the GluN1 subunit was measured. The GluN1 receptor expression (shown as relative optical density) in the TSA + O/S group was significantly higher compared with the Sal + O/S group (Fig. 9, $p< 0.01$). In addition, when examining non normalized data, the GluN1 receptor subunit was significantly down-regulated in Sal+O/S compared with Sal + O/O groups ($t= 7.795$, $p < 0.0001$) which is in keeping with a previous study (Lethbridge et al., 2012). All data were normalized with the Sal +Odor group. Therefore, this result suggests that odor-stroke conditioning leads to GluN1 receptor down-regulation in vehicle-treated groups but not in TSA-treated groups. Down-regulation of GluN1 at 3 h after initial early odor learning influences the effects of a second training event at the same synapse. HDAC inhibition

prevents GluN1 down-regulation, blocking unlearning and maintaining memory at 21 h after a second session of odor preference training.

CHAPTER – 4 DISCUSSION

4.1 Summary of major findings

I found that HDAC inhibition by TSA led to memory extension in an odor preference learning model. This prolonged memory lasted at least 9D after conditioning. I also examined the relation between GluA1 membrane localization and memory extension. The western blot experiments suggest that the expression of GluA1 in the synaptic + extrasynaptic membrane fraction was increased at 48 h after training and this increased GluA1 expression was also observed immunohistochemically in the glomerular layer 5D after conditioning. Interestingly, the increased GluA1 expression coincides with odor preference memory at 5D after training.

I also found that prolonged memory was specific to the paired odor and the TSA itself remained effective for less than 24 h following intrabulbar infusion. Next, I tested the role of HDAC inhibition in a GluN1-mediated unlearning paradigm. In the one-trial early odor preference learning model, GluN1 is normally downregulated 3 h after training and this downregulation of GluN1 induces memory impairment at 21 h if odor preference re-training is given at that 3 h time point. However, infusion of TSA prevented GluN1 downregulation at 3 h after training and abolished the unlearning after re-training at that time point.

4.2 Qualitative immunohistochemical results of histone acetylation

Qualitative immunohistochemical data confirmed that TSA was infused in the right place of the OB. This was shown in the odor only trained pups where TSA-infused olfactory

bulbs showed increase histone acetylation relative to vehicle-infused olfactory bulbs (Fig. 1B). These qualitative results also confirmed the basic concept that HDAC inhibition can be involved in one or more subtypes of histone acetylation in the OB. Visual inspection using light microscopy revealed no detectable differences in histone acetylation between two OBs of the odor + stroke animal (TSA-treated vs. vehicle-treated side, Fig.1A). Therefore, it is very difficult to identify the detectable changes in histone acetylation in this qualitative study. The possible reason is that TSA and stroking both are involved histone acetylation. During HDAC inhibition, TSA blocks an HDAC enzyme and helps indirectly in the histone acetylation via DNA relaxation whereas stroking itself can also be involved in histone acetylation. But the molecular mechanism of histone acetylation via HDAC inhibition and stroking is not well-known.

However, interestingly, an increased level of acetylation of H3 and H4 was observed in the TSA-treated OBs compared with vehicle-treated OBs in odor only (ie. non-learning) trained pups at both the 30 min and 2 h time points (Fig. 1B). Though an increased level of histone acetylation was observed qualitatively in the TSA-treated OBs compared with vehicle-treated OBs in odor only trained pups, there was no learning effect observed in TSA+O/O animals behaviourally. During olfactory training, odor by itself cannot induce norepinephrine release from the locus coeruleus which is very important for calcium influx mediated downstream gene regulation whereas odor-stroke (O/S) training induces norepinephrine release mediated downstream gene cascade activation (Rangel and Leon, 1995). In summary, from this qualitative immunohistochemical analysis, it appears that histone acetylation is increased in learning & non-learning conditions and my behavioral data

show histone acetylation, by itself, is not necessary for extended memory. Thus, some other factor or factors must be involved in the molecular mechanisms leading to extended memory, perhaps via histone acetylation influence on pCREB (Strong, 2014) or other downstream genes.

4.3 Models for extended memory

A German psychologist, Hermann Ebbinghaus, discovered a way to improve learning. He carried out the first recorded experimental studies on human memory. In his studies, he found that increased repetition during a period of study obviated the need to relearn and also helped subjects to remember that session 24 h later. But, a major flaw of this study was that Ebbinghaus was the only subject in his study. This limited the study's generalizability to the population (Ebbinghaus, 1913).

Since then many researchers have used multiple training sessions to extend memory. As one example, four trials a day for 6 days in a Morris water maze produced extended memory which was evident 14 days after spaced training (Spreng et al., 2002).

Researchers investigated how memory could be extended for longer time periods when using a single training session. In water maze experiments, animals trained with massed or spaced trials initially **learn** similarly, but more of those trained with **spaced** trials **remember** the location of the water maze platform 2 weeks later (Scharf et al., 2002). In contrast, animals trained with single trial in the water maze only remembered for a maximum of 24 h. In our lab we utilize a single trial early odor preference learning model in which 10 min of training on PND 6 with stroking and odor induces a 24 h odor preference memory in neonates. This single trial odor preference memory only lasts for

24 h, but not for 48 h (McLean et al., 2005).It required four separate daily sessions to yield 48 h memories or longer (Fontaine et al., 2013).

In investigating the intracellular correlates of shorter and longer duration long-term memories it would be ideal to modulate a single trial memory pharmacologically such that comparisons of protein transcription and translation could be made following both a training that results in 24 h memory and the same training when it leads to multiday memory.

Previously, our lab has manipulated cAMP cascade activation itself combined with single trial odor preference training to extend memory. For example, the β-AR agonist, isoproterenol (2mg/kg) in combination with the PKA agonist (Sp-cAMP) enhances memory by extending normal 24-h retention to 48–72 h (Grimes et al., 2012). In other experiments from our lab isoproterenol (1mg/kg) in combination with the phosphodiesterase IV (PDE4) inhibitor, cilomilast extended memory to 4 days (McLean et al., 2005). Cilomilast prevents cyclic-AMP breakdown extending CREB phosphorylation. However, the normally effective dose of isoproterenol (2mg/kg) along with PDE4 was unable to induce prolonged memory. It was speculated that the weaker dose of β-AR agonist (1 mg/kg isoproterenol) in combination with cilomilast helps to maintain a better balance of kinase/phosphatase signaling. While the normally effective dose of β-AR agonist along with cilomilast appears to disrupt the balance of kinase/phosphatase signaling (McLean et al., 2005). Most recently studies from the McLean lab have revealed a role for HDAC inhibition in mediating memory extension: isoproterenol (1mg/kg), cilomilast (3 mg/kg) and the HDAC inhibitor, sodium butyrate (NaB; 1.2 g/kg) were injected subcutaneously to extend olfactory preference memory.

This Iso+Cilomilast+NaB combination extended memory up to 5 days after training but the combination of Iso+NaB (without cilomilast) was unable to extend memory (Strong, 2014). Most of these aforementioned studies used pharmacological cocktails to prolong odor preference memory. However, a recent study suggested that intrabulbar TSA infusion alone combined with odor-shock training could enhance aversive memory even 2 days after training (Wang et al., 2013).

In my experiments, I established a model in which odor preference memory was extended for at least 9D after one trial on PND 6 consisting of 10 min odor + stroking training combined with a single intrabulbar infusion of TSA prior to training.

4.4 Relation between long-lasting memory and GluA1 expression

Several researchers suggest that increased glutamatergic AMPA receptors are the substrate for long-term memory (Lynch and Baudry, 1984; Lu et al., 2001; Malinow and Malenka, 2002). Previous study has shown that increases in the AMPA receptor subunit GluA1 is a prime candidate for the underpinning of LTP (Selcher et al., 2012). Membrane associated AMPA receptor subunit GluA1 was increased in the amygdala 24 h after fear conditioning (Yeh et al., 2006). The glomerular layer of the OB, which receives odor input, is likely to have a crucial role in the synaptic changes that underlie peppermint preference learning (Yuan et al., 2002). The DL quadrant of the glomerular layer, in particular, is responsive to several compounds including carvone, a key component of peppermint extract (Diaz-Maroto et al., 2008), acetophenone, eugenol and in the DM quadrant, glomerular layer responses are activated by compounds which include organic acids (Bozza and Kauer, 1998; Kikuta et al., 2013). According to these studies, these two quadrants of the

glomerular layer (DL and DM) may be critical for peppermint odor preference learning. In 2011, Cui et al. showed that membrane GluA1 levels were increased in the OB glomerular layer 24 h after conditioning in the appetitive odor preference learning model, but this increase was no longer observed 48 h after training. In addition, odor preference behaviour or memory was not expressed 48 h after conditioning (Cui et al., 2011). These results suggest that changes in GluA1 expression correlate with memory retention. Further in the Cui et al. studies, blockade of GluA1 increases prevented odor preference learning arguing for a causal role for GluA1 increases in mediating odor preference learning.

Present results in the TSA- induced prolonged odor preference memory model show that GluA1 increases 48 h after training using western blot technique and remains elevated in the glomerular layer of the OB at least 5D after odor preference training. In this study, western blot technique was unable to show the significant difference of GluA1 expression 5D after training. Western blot method used synaptic + extra synaptic membrane fraction of the whole OB. Therefore, it is not surprising that the increased GuA1 expression was not seen using western blot from the membranous fraction of whole OB. Whereas, IHC is an excellent detection technique and has the advantage of being able to show exactly where a given protein is located within the tissue, the IHC experiment found the increased GluA1 expression was localized in the DL and the DM quadrant of glomerular layer 5D after training, when odor preference memory is also expressed behaviourally. This is consistent with a role for increased excitatory strength in these glomeruli in mediating odor preference behaviour. In summary, current results are consistent with the AMPA receptor hypothesis of long-lasting memory and provide a strong correlation between GluA1

expression in the glomerular layer and memory extension.

4.5 Functional significance of the unlearning mechanism

The unlearning is a cognitive process that helps to discard specific learned memory from the brain. The term ‘unlearning’ is still controversial, and the molecular difference with memory forgetting and memory extinction processes is not well defined. Experimentally, repeated exposure to the conditioned stimulus without providing the unconditioned stimulus can produce extinction of previous memory (Berman and Dudai, 2001; Bouton, 2004; Mickley et al., 2010), whereas failure to remember or forgetting is a natural phenomenon of the brain. In the case of unlearning, both the conditioned stimulus and the unconditioned stimulus are present but the memory is still abolished (Bouton, 2002). Behaviorally, it is shown that retraining at the 3 h time window can produce unlearning. The functional significance of unlearning in our daily life remains unsolved. According to Klein et al., unlearning is the mechanism of practice change (Klein, 1989). Hedberg’s hypothesis suggests that unlearning is the conscious discarding of some memories (Hedberg, 1981). In a very recent study, scientists showed that unlearning can reduce gender and racial bias (Hu, 2014).

Researchers have suggested that if we understand the process of unlearning we will be able to understand how to unlearn criminal activity, racial and gender bias related activities (Vanes et al., 2014). In my current work, I found that infusion of TSA prior to initial training abolished unlearning following a re-training at 3 h. The previous model of unlearning helped us understand the mechanisms involved in the unlearning process (Mukherjee et al., 2014). Now with this new prolonged memory model, it will be easier to

compare the unlearning state and the learning state after retraining. These data demonstrated that downregulation of NMDA receptors is prevented by strong learning, which reduces the likelihood of forgetting. They also suggest that the specific NMDA receptors involved in unlearning are the only ones targeted by TSA induced memory strengthening protocol.

4.6 Conclusions and future directions

This study provides a single trial extended odor preference memory model. This prolonged memory is related to GluA1 membrane expression. Immunohistochemical data suggest that GluA1 expression occurs selectively in the DL and DM quadrants of the GL but not in the associated EPL. This extended memory is specific to the paired odor. I also showed that TSA appeared to be only effective on the day of infusion. However, further experiments showing training with orange plus stroking stimulus in the presence of TSA induces 3D memory would strengthen this conclusion. On the other hand, other experiments in our lab show that TSA induces histone acetylation for only around 30 min so the acetylation effect of TSA does not appear to last long (Strong, 2014). Normal GluN1 mediated unlearning is not observed in the single trial extended odor preference memory model, which would support the HDAC inhibition role in memory strengthening and a reduction in the implementation of possible forgetting mechanisms.

To date, there is no other model that shows 9D extended odor preference memory following a single 10 min training trial. Thus, these studies are the first to show the effectiveness of TSA in producing such a prolongation of memory, and especially of appetitive memory. This unique model will help to illuminate the mechanisms for long

lasting preference memory in the future.

Future research arising from this thesis work should focus on the potential molecular mechanisms for HDAC inhibition mediated memory extension and increased GluA1 expression that is maintained even at 5D after training. In early odor preference learning, rat pups are trained with 10 min odor+stroking training. This training activates the cAMP/PKA signaling cascade leading to an increase in CREB phosphorylation 10 min after training and produces 24 h long-term memory. However, a 10 min single training trial produces memory that is seen at 24 h, but not at 48 h. It is still unclear what events/substrates differentiate a memory lasting 24 h from a memory lasting beyond 24 h (extended long-term memory). According to the literature, cAMP/PKA/CREB signaling cascade activation and HDAC inhibition-mediated gene transcription both are critical in extended memory formation. Previous work has demonstrated that HDAC4 and HDAC5 are highly enriched in the brain (Kim et al., 2012). A study indicated that HDAC4 knockout mice showed long-term memory impairment, but HDAC5 knockout mice did not. Therefore, HDAC 4 is a positive regulator of learning and memory, while HDAC5 appears to be a negative regulator of learning and memory (Kim et al., 2012). In 2010, an *in-vitro* study showed that PKA phosphorylates HDAC5 at serine 280 and prevents nuclear export of HDAC5 (Ha et al., 2010). This HDAC5 leads to the inhibition of gene transcription (Ha et al., 2010). On the basis of this literature, I hypothesized that during odor+stroke training, PKA induces HDAC5 phosphorylation and inhibits nuclear export of HDAC5 as well as playing a role in CREB phosphorylation. As a result, extended gene transcription may be prevented by HDAC5. For this reason, cAMP/PKA signaling mediated CREB phosphorylation might be sufficient for 24 h long-term memory, but not

for 48 h or extended memory. In my current model I used TSA, an HDAC inhibitor. It is possible that PKA is only involved in CREB phosphorylation for 24 h memory but TSA enables DNA to become more accessible for gene transcription and longer memory. That gene transcription may be enough to induce the critical elements for at least 9D memory formation. To test this hypothesis PKA mediated HDAC5 phosphorylation at serine 280 could be assessed by western blot techniques in the normal odor + stroke 24 h long-term memory model compared to the TSA-induced extended preference memory model. I expected that PKA mediated HDAC5 phosphorylation at serine 280 would be increased in the 24 h long-term memory model but not in the TSA induced extended preference memory model. Unfortunately, a reliable phosphorylated HDAC5 S280 antibody was not commercially available.

I also tried to test my hypothesis indirectly. Previous study demonstrated that nuclear Ca2+/calmodulin-dependent protein kinase II (CAMKII) phosphorylates HDAC5 at serine 259 and 498 and helps to export HDAC5 from the nucleus to the cytoplasm (Ha et al., 2010; Schlumm et al., 2013). To assess phosphorylation of HDAC5 at two different serine residues (S259 and S498) in the 24 h normal odor+stroke model and also in TSA induced extended memory model in the cytoplasm, I used two different antibodies with western blot techniques (phospho HDAC5 serine 259, Sigma, St. Louis, Missouri USA, cat. no.SAB4503878 and phospho HDAC5 serine 498, Abcam, Cambridge, MA, USA, cat. no.ab47283). I expected that phosphorylated HDAC5 expression would not be increased in cytosol of the TSA induced extended memory model. This experiment would have provided indirect evidence for my hypothesis about mechanisms. Unfortunately, neither of these antibodies worked well. Investigation of direct interaction between PKA

and HDAC5 by using a co-immunoprecipitation (Co-IP) technique may be another way to test this hypothesis in the future.

There is another question still unsolved that relates to learning using natural means (non-drug induced memory enhancement). That is, how does DNA become more accessible for a longer time period and help in extended memory formation in the absence of drug-induced HDAC inhibition? For this question, my hypothesis is that during single trial long lasting memory formation in a natural system (non-drug induced memory enhancement), a high amount of calcium influx helps to increase nuclear calcium. This nuclear calcium activates nuclear CAMKII which further phosphorylates HDAC5 at two different serine residues (S259 and S498) and plays a role in export of HDAC5 from nucleus to cytoplasm. This nuclear export of HDAC5 promotes DNA relaxation for a longer time period and permits PKA mediated full gene transcription. This pathway may represent a possible mechanism by which cAMP/PKA/CREB signaling cascade can extend memory for a longer time frame without pharmacological HDAC inhibition.

In our 24 h odor preference memory model, re-training related unlearning is possibly mediated by 3 h GluN1 downregulation. Interestingly, this GluN1 downregulation mediated unlearning is not observed in the extended odor preference memory model using TSA. Therefore, a next step could be to identify the possible molecular pathways behind it. It is also necessary to find out more about the unlearning mechanism in the natural system, because at present we don't have any broad ideas about the unlearning mechanism in our daily life, although the Drosophila studies suggest it may play a role in natural forgetting (Berry and Davis, 2014).

Future experiments should aim to characterize memory related gene expression in the OB

for the TSA induced extended odor preference memory model. This will help to identify genes that are differentially regulated by TSA and likely to be critical for memory extension. Also, future experiments could examine the expression of GRIA1 (glutamate receptor, ionotropic, AMPA 1), a GluA1 protein coding gene, after TSA infusion in the TSA induced extended odor preference memory model. The present data suggests expression of the protein is extended in time and should be appropriately supported by increased gene transcription or translation. This experiment can be done through microarray studies and it would help to further establish a clear relationship between GluA1 gene expression and memory extension. We can do other experiments to establish the causal relationship of AMPA expression and extended memory by using Tat-GluA1 carboxyl tail (Tat-GluA1$_{CT}$) interference peptide prior to TSA infusion in the extended memory model. This interference peptide specifically blocks the endocytosis of AMPA receptors (Yu et al., 2008).

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Figures – RESULTS

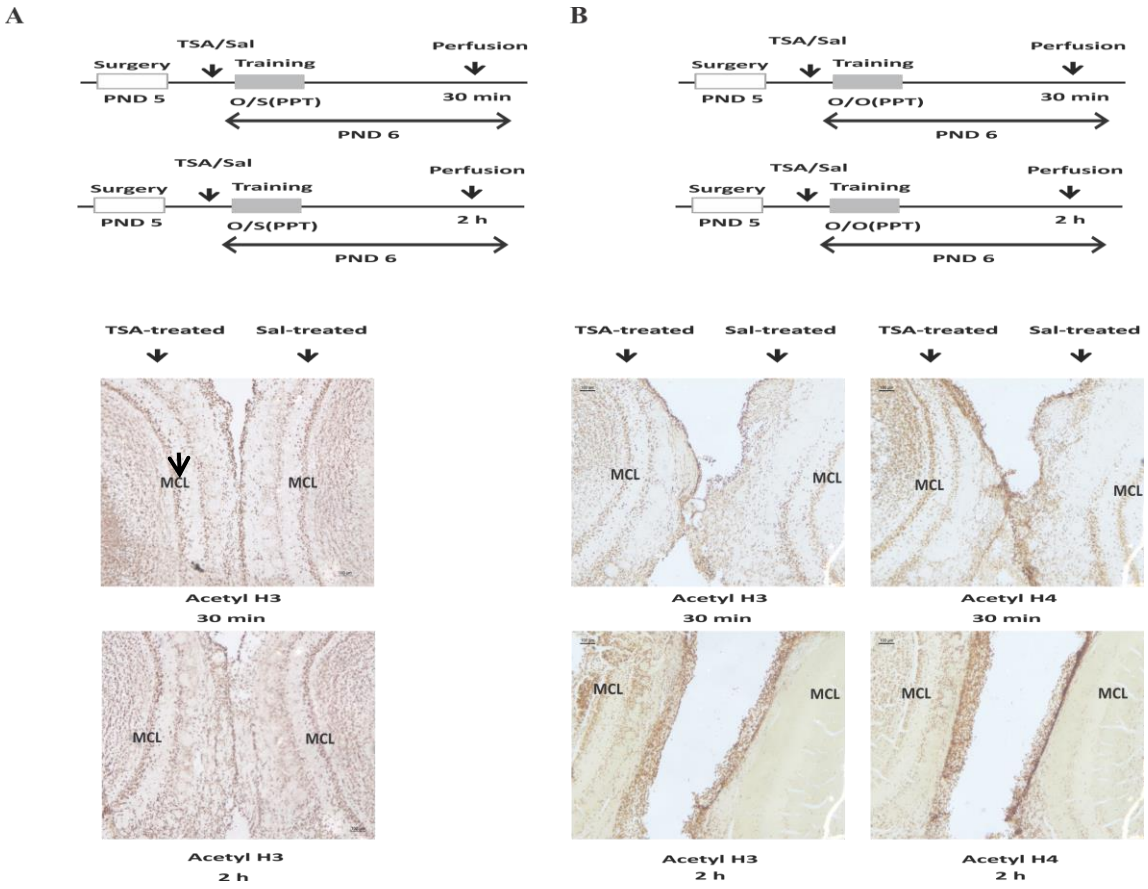

Figure 1: **Representative immunohistochemical localization of AcH3 and AcH4 in the olfactory bulb of PND 6 pups sacrificed 30 min or 2 h following odor preference training**. **A)** AcH3 expression at 30 min and 2 h after TSA induced O/S training.**B)** AcH3 and AcH4 expression at two different time points (30 min, 2 h) after TSA induced O/O training.

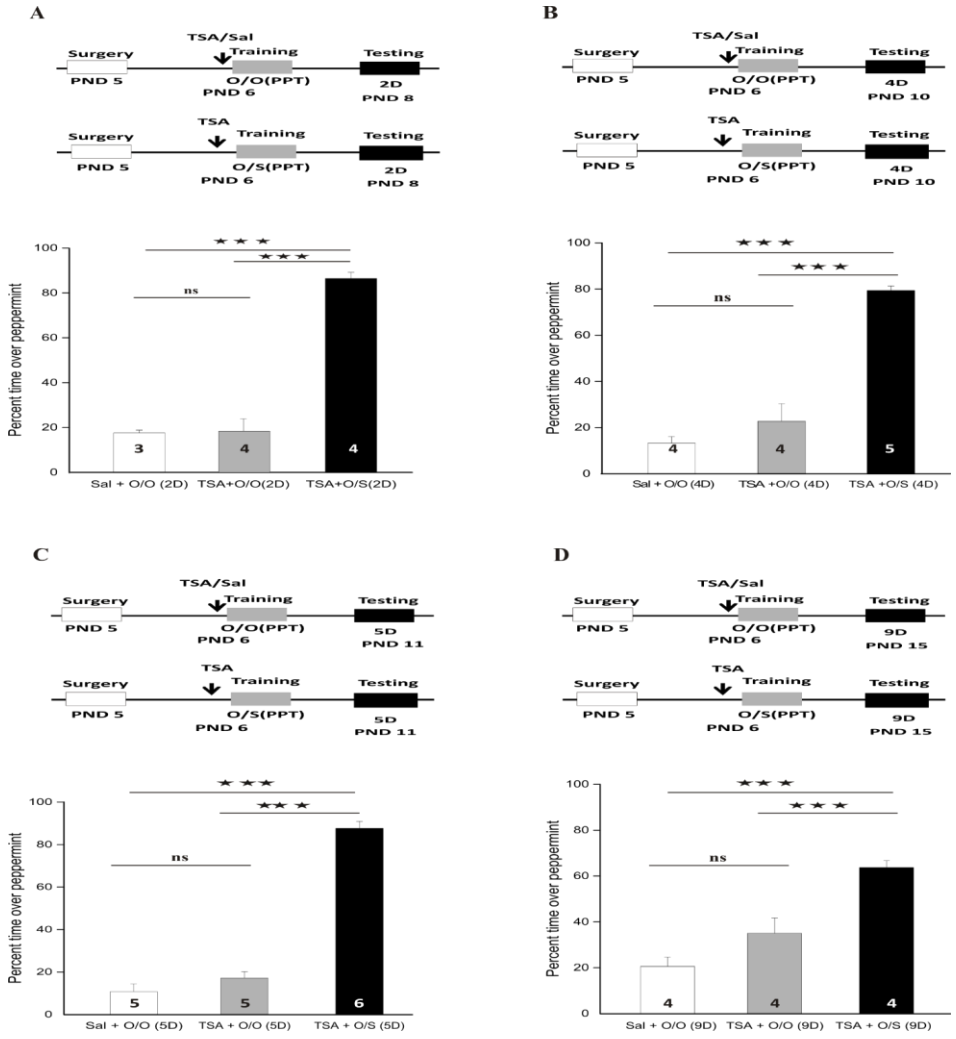

Figure 2: **HDAC inhibition induces odor preference memory extension.** One-way ANOVA with a Tukey's post hoc test was performed to analyze the behavioral data. **A)** Behavioral data show the significantly robust memory in neonatal rat pups 2D post-training (2D: $F_{(2, 8)} = 102.42$; ***$p < 0.001$). **B-D)** Time course analysis of preference behavioral studies reveals a significant higher memory in TSA-treated groups relative to control groups at 4D,5D and 9D after odor preference training (4D: $F_{(2, 10)} = 67.699$; ***$p < 0.001$; 5D: $F_{(2, 13)} = 169.31$; ***$p < 0.001$; 9D: $F_{(2, 13)} = 169.31$; ***$p < 0.001$).

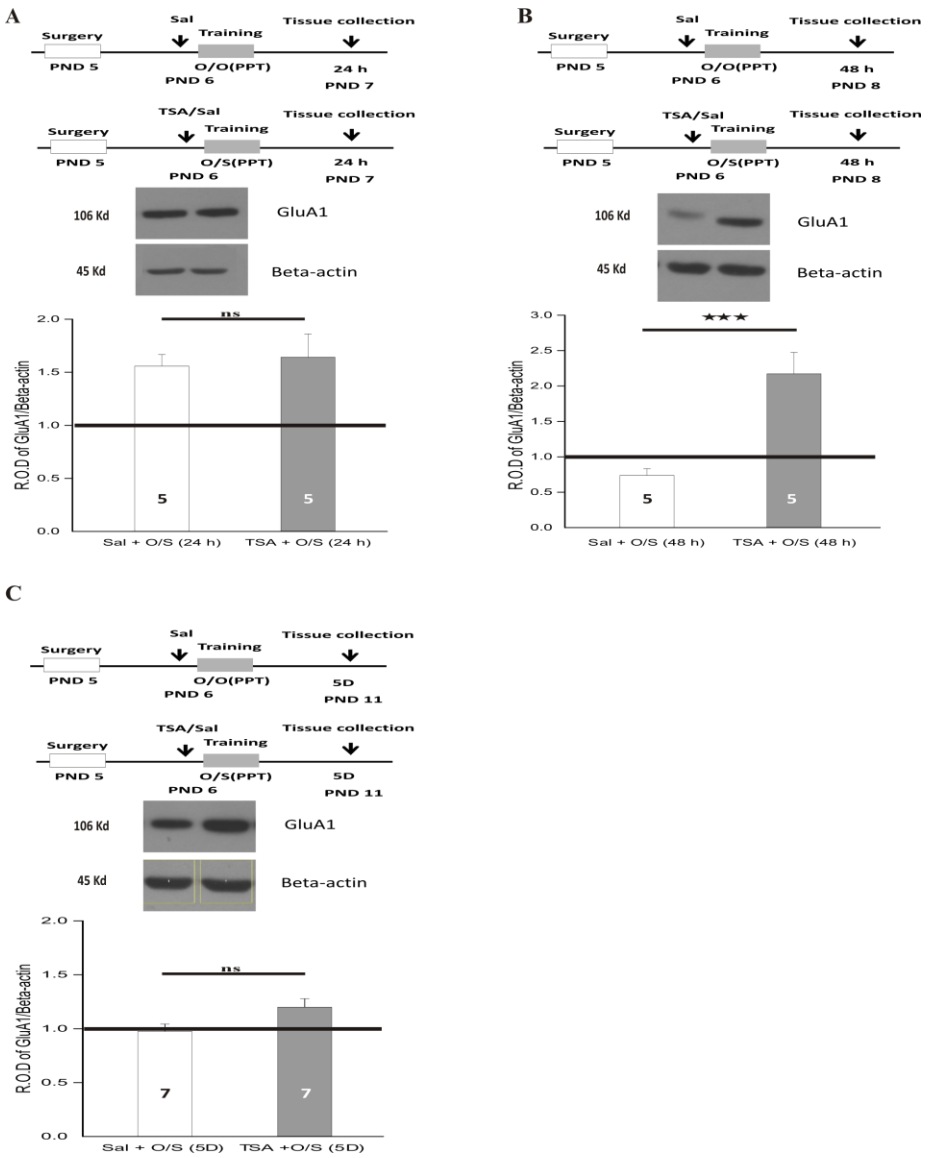

Figure 3: **Western blots of GluA1 expression showing the relative optical density of GluA1/Beta-actin (mean ± SEM) in neonatal rat pups at 24 h, 48 h and 5D post-training.** All data are normalized to Sal +Odor control groups (represented as a horizontal line). Representative bands and experimental protocol are above each graph. In western blot experiments, GluA1 expression was analyzed by two-way ANOVA followed by Tukey's post hoc comparisons with both time and groups as factors ($F_{(1, 28)} = 21.44$). **A)** Time course analysis of GluA1/Beta-actin in rat pups shows no significant difference in

the TSA–treated group compared with the Sal+O/S group 24 h after training ($p > 0.05$). **B)** Whereas, 48 h post-training reveals a significantly higher GluA1 expression in TSA–treated groups relative to the Sal+O/S group (***$p < 0.0001$). **C)** GluA1 expression in the TSA + O/S group did not show any significant difference compared with the Sal+ O/S group 5D following training ($p > 0.05$).

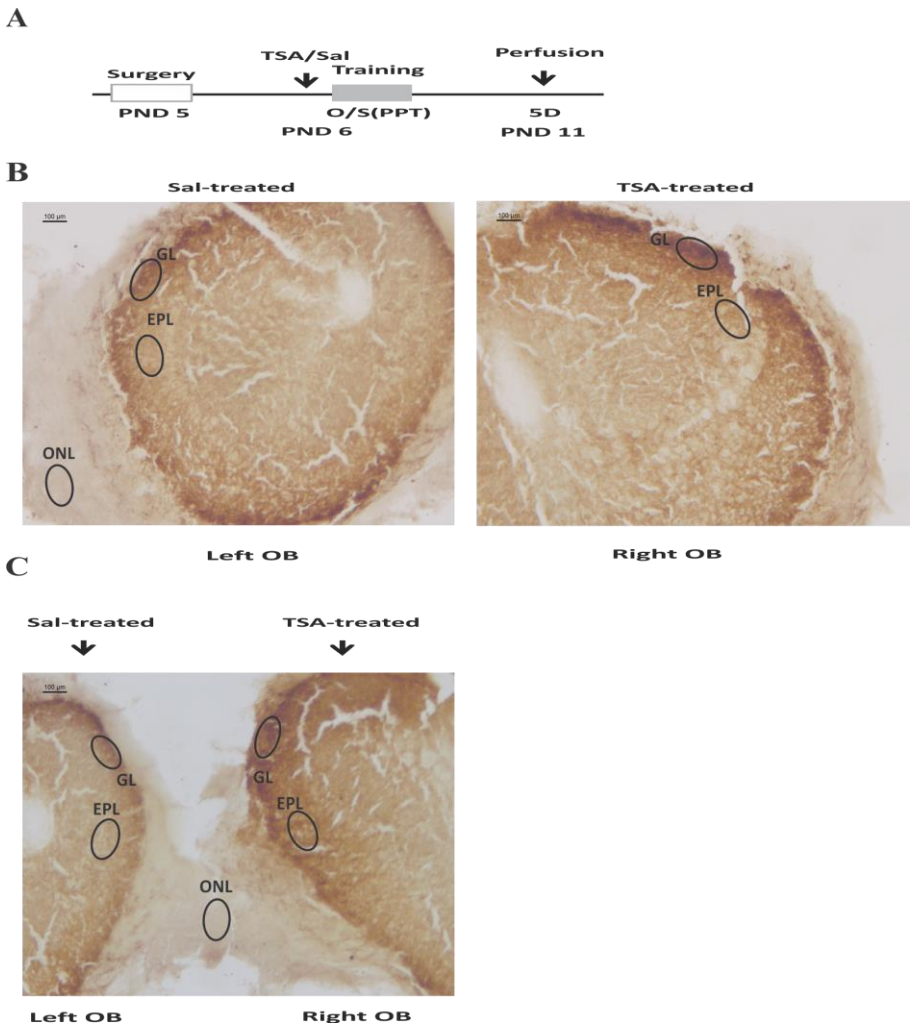

Figure 4: **Immunohistochemical (IHC) expression of GluA1 in the glomerular and external plexiform layer of the olfactory bulbs 5D after training.** Representative immunohistochemical localization of GluA1 (TSA-treated Right OB vs. Vehicle-treated left OB) in different quadrants of the OB **A)** Experimental protocols are at the top. **B)** Left and right OB dorsolateral quadrant of the glomerular layer (DL-GL) and external plexiform layer (DL-EPL) **C)** dorsomedial quadrant of glomerular layer (DM-GL) and external plexiform layer (DM-EPL) of the olfactory bulb in intra-animal-infused PND 11 pups sacrificed 5D after odor preference training. B-C is at the same magnification as the bar in B (100 μm).

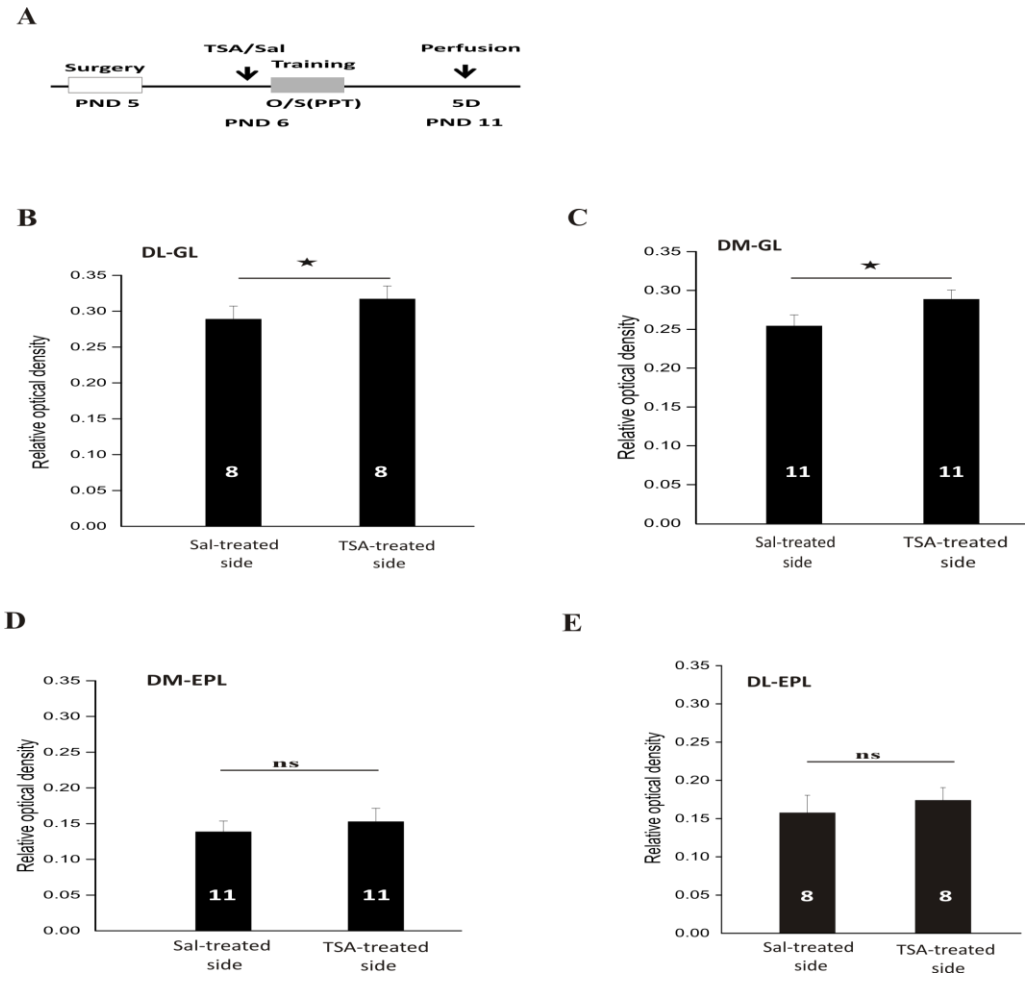

Figure 5: **Quantification of expression of GluA1 in the glomerular layer (GL) and external plexiform layer (EPL) of the olfactory bulbs 5D after training.**

A) Experimental protocol is at the top. Graphs showing the density GluA1 expression in various regions including: **B)** dorsolateral quadrant of the glomerular layer (DL-GL), **C)** dorsomedial quadrant of the glomerular (DM-GL) , **D)** dorsomedial quadrant of external plexiform layer (DM-EPL) and **E)** dorsolateral quadrant of external plexiform layer (DL-EPL) of the OB. Paired two-tail t-tests revealed significantly higher expression of GluA1 is observed in the DL-GL and DM-GL layers of TSA-treated right OB relative to vehicle-treated left OB (dorsolateral: $t_{(7)} = 2.962$; *$p < 0.05$; dorsomedial: $t_{(10)=} 2.85$; * $p < 0.05$). However, no significant difference is observed in comparisons of within animal treatments in the DM-EPL and DL-EPL layer.

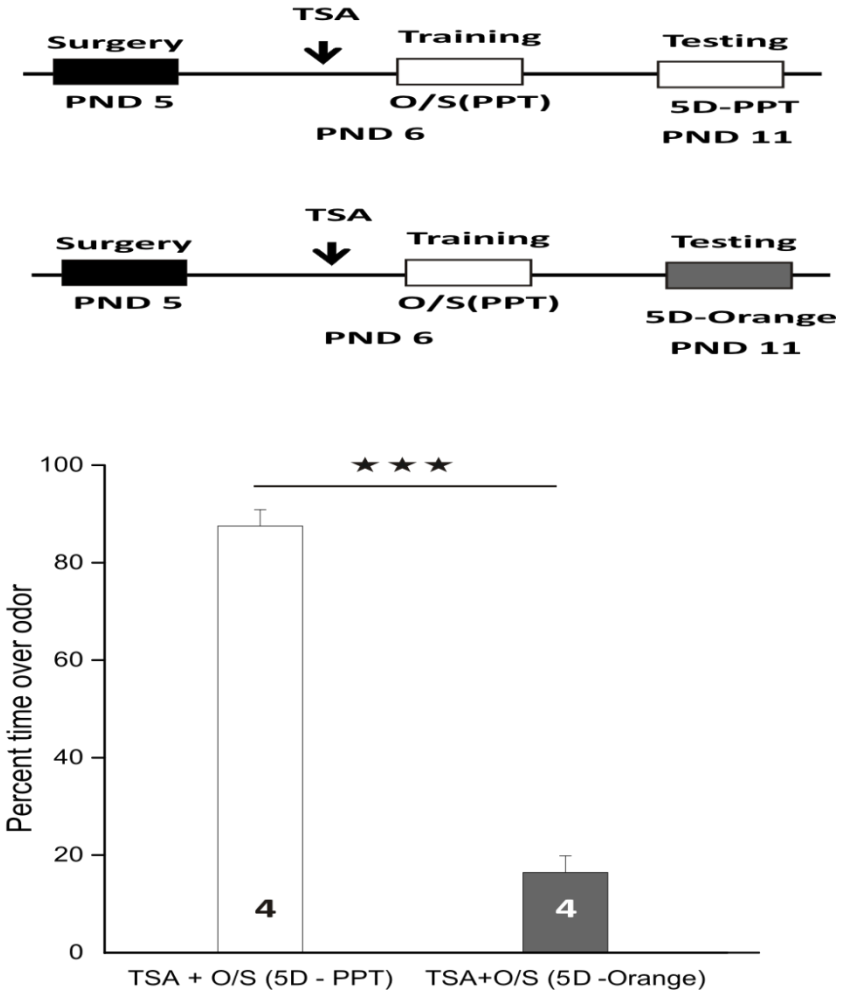

Figure 6: **TSA induced odor preference memory is specific to paired odor.** TSA induced odor preference memory is specific to paired odor. Behavioral data reveal the time spent over peppermint is significantly higher compared to the time spent over orange. The experimental protocol is at the top. An unpaired two-tailed t-test was used to evaluate the two group comparisons ($t_{(6)} = 2.508$; ***$p < 0.0001$).

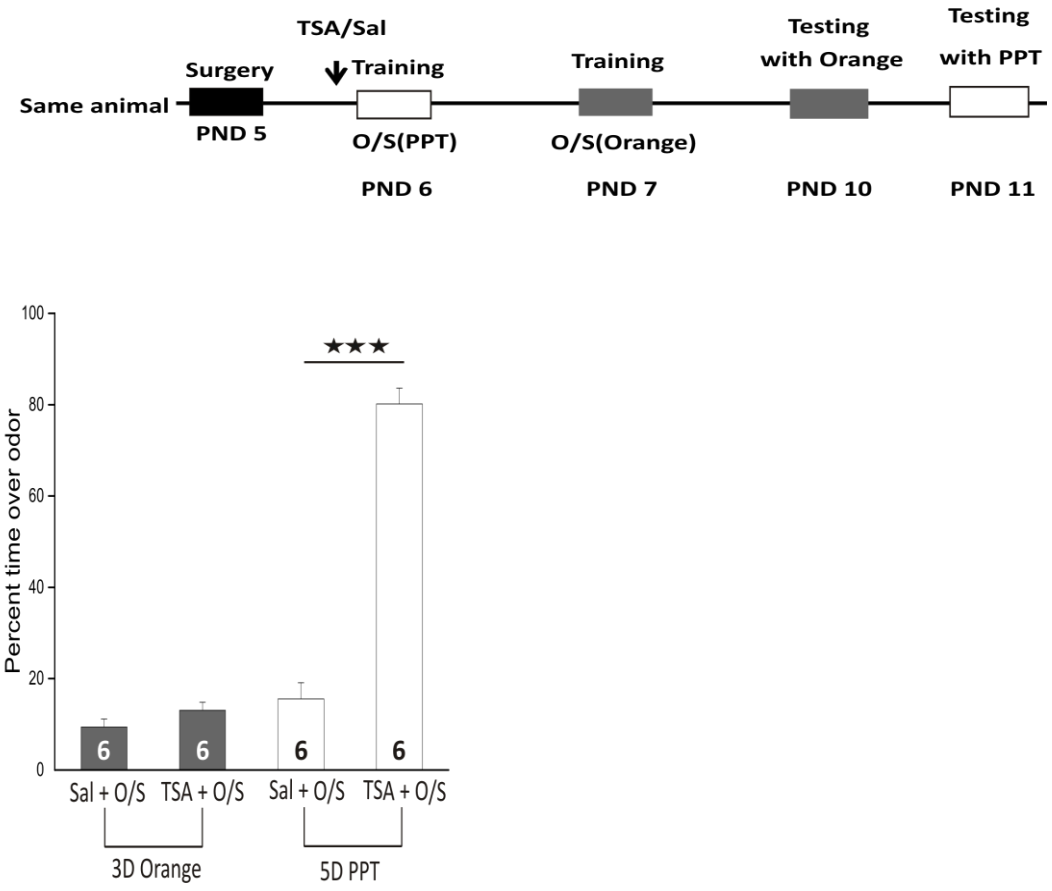

Figure 7: **How long does TSA remain effective?**

The behavioral protocol is shown above the graph. The graph shows that the TSA-treated pups spent significantly more time over the peppermint than the vehicle-treated pups. But vehicle-treated and TSA-treated pups did not show any preference for orange. A mixed factorial designed ANOVA was used to compare the groups (TSA-treated vs. saline-treated) and a between-subjects factor and conditions (time spent over orange at 3D vs. time spent over peppermint at 5D), as a within-subjects factor. This analysis revealed both a significant main effect of condition ($F_{(1, 10)} = 235.23$; ***$p < 0.0001$) and a significant condition by group interaction ($F_{(1, 10)} = 162.69$; ***$p < 0.0001$).

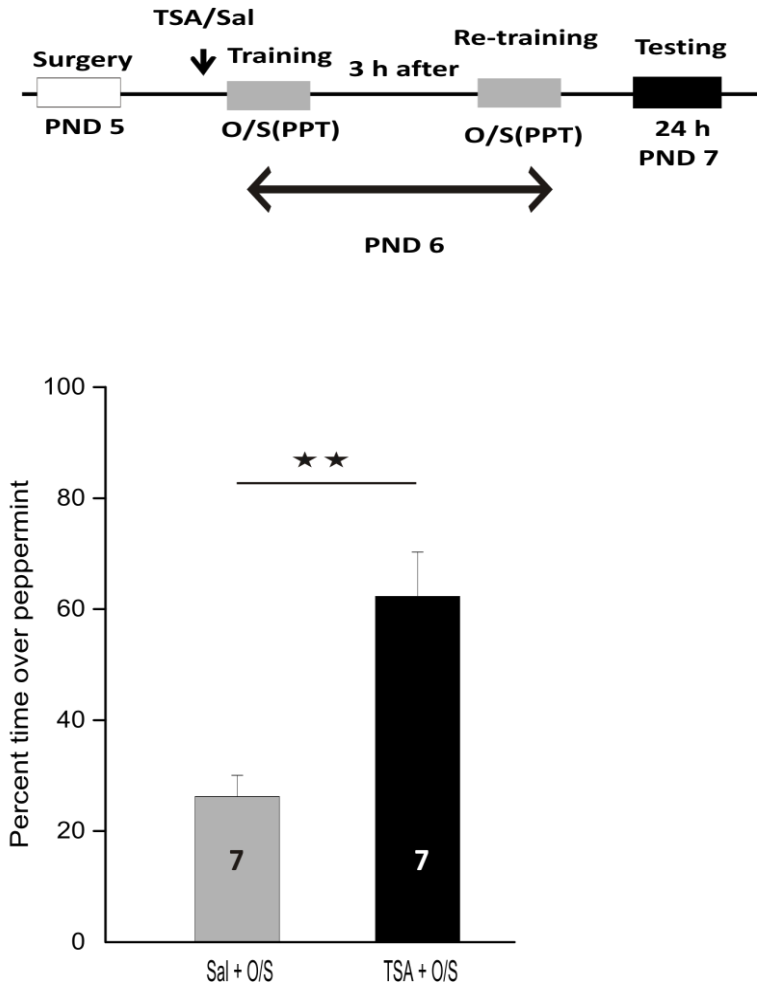

Figure 8: **TSA can abolish unlearning in pups retrained at 3 h.** The experimental protocol is shown above the graph. Behavioural data show that odor-stroke re-training at 3 h interferes with the expression of the odor preference induced by the initial exposure in the vehicle-treated odor-stroke group but not in the TSA-treated group ($t_{(6)=}$ 4.079; **p< 0.01).

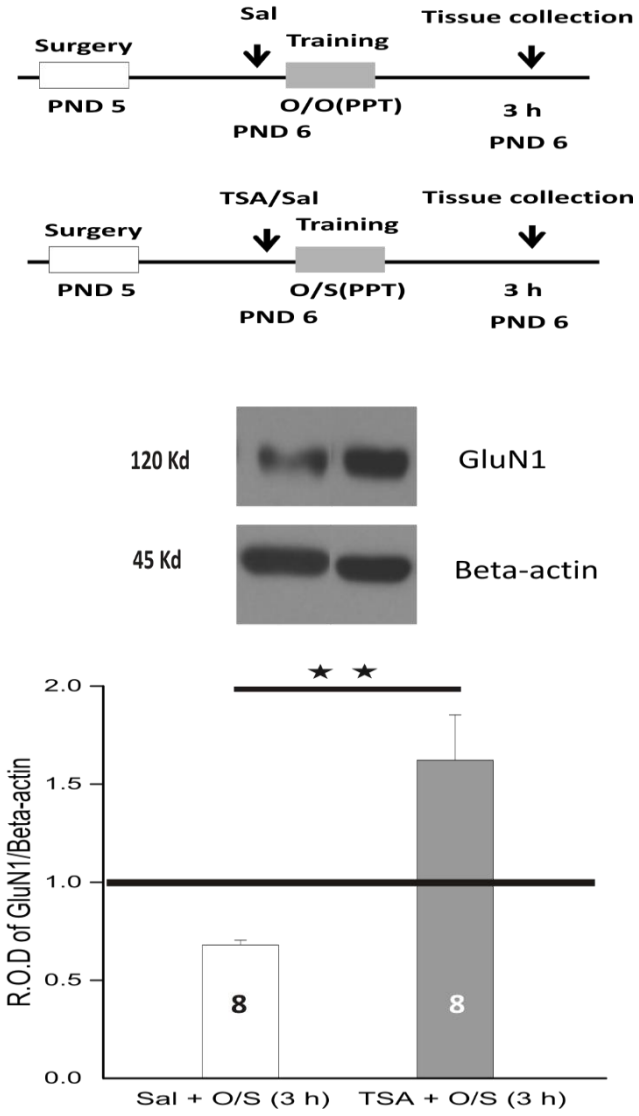

Figure 9: **GluN1 down-regulation occurs in vehicle-treated pups at 3 h but not in TSA-treated pups.** Western blots of GluN1 expression show the relative optical density of GluN1/Beta-actin (mean ± SEM) in the olfactory bulbs at 3 h post-training. All data are normalized to Sal +Odor control groups which is represented as a horizontal line. Representative bands and experimental protocol are at the top. GluN1/Beta-actin shows a significant difference in TSA-treated pups compared with Sal+O/S treated pups 3 h after training, revealed by unpaired two-tailed t-test ($t_{(7)=}$ 4.026; **p< 0.01).

APPENDIX 1: Protein Extraction Recipes

Buffered Sucrose (20mL)

Sucrose	2.2g
1M Tris pH 7.4	200µl
0.5 M EDTA	40µl
50mM EGTA	400µl
10x mini protease inhibitor	2 pellets
10x phosphatase inhibitor	2 pellets
Distilled water(dH_2O)	Top up to 16 ml

Buffered 10x STE (20mL)

1M Tris pH 7.4	2mL
0.5 M EDTA	400µl
SDS	2g
10x mini protease inhibitor	2 pellets
10x mini phosphatase inhibitor	2 pellets
Distilled water (dH_2O)	Top up to 16 ml

Add 1ml 10x STE in 9ml distilled water (final volume 10 ml) to make 1x STE buffer on day of use.

APPENDIX 2: Western Blotting Recipes

Tris pH 6.8 (0.5 M) 50 ml

Tris Base	3.029g
dH2O	40 ml

Adjust pH to 6.8

Tris pH 8.8 (1.5 M) 50 ml

Tris Base	9.086 g
dH_2O	40 ml

Adjust pH to 8.8

Running Buffer (10x) – Stock Solution

	For 500 ml	For 1L	Final concentration
Tris Base	14.14 g	30.3 g	250 mM
Glycine	72 g	144 g	1.92 mM
SDS	5.0 g	10 g	1.00%

pH should be 8.8 Use 1x on day of use.

Transfer Buffer (10x) – Stock Solution

	For 500 ml	For 1L	Final concentration
Tris Base	15.14 g	30.3 g	250 mM
Glycine	72 g	144 g	1.92 mM

pH should be 8.3. Use 1X on day of use (700 ml dH_2O, 200 ml Methanol, 100 ml Transfer Buffer 10x).

TBST pH 7.5

	Working concentration	For 2L(5X)	For 2L(10X)
NaCl	137 mM	80 g	160 g
KCl	2.7 mM	2 g	4 g
Tris Base	25 mM	30 g	60 g
Tween 20	0.1%	10 ml	20 ml

Adjust pH to 7.5 and make up volume to 2 L

Sample Buffer (5x)

Glycerol	5 ml
SDS	1 g
Bromophenol Blue	25 mg
Tris 1.0 M, pH 6.8	3 ml

Adjust pH to 8.8 at 25^{o}C.

On day of use

5x Sample Buffer	200 µl
Dithiothreitol (DTT)	15.4 mg

Warm 5x sample buffer to dissolve.

Blocking reagent (5% non-fat dry milk)

TBST	10 ml
Powdered non-fat dry milk	0.5 g

	4% staking gel	7.5%	10%
Acrylamide/BisAcrylamide 30% 29:1	650 µl	2.5 ml	3.3 ml
dH_2O	3.3 ml	4.25 ml	3.4 ml
Tris, 1.5 M pH 8.8		2.5 ml	2.5 ml
Tris 0.5 M pH 6.8	630 µl		
SDS 20%	100 µl	50 µl	50 µl
APS 1.5%	332 µl	700 µl	700 µl
TEMED	8 µl	5 µl	5 µl