DISSECTING THE REGISTRATION AND PROCESSING OF OLFACTORY EVENTS DURING GENERAL ANESTHESIA

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It is commonly accepted that general anesthesia produces unconsciousness and amnesia. However, the extent to which the anesthetized brain receives and retains information is not well understood. Memory of a given event relies upon initial consolidation and subsequent retrieval. In order to test the effects of anesthesia on these separate memory components, we compared what rats reported behaviorally with what their brains 'reported' histologically.

In chapter 2, we fully anesthetized rats with ketamine/xylazine anesthesia and exposed the animals to a novel odorant mixture. The following day the rats were presented with the same odorant mixture while awake in order to assess behavioral familiarity. The rats demonstrated no memory of the odorants previously presented during anesthesia. In order to assess odor-induced brain changes, rats were sacrificed for histological analysis 2 hours after exposure to the odorants during anesthesia, or 2 hours after re-exposure while awake the following day. We quantified the protein expression of *c-fos*, an immediate early gene thought to be critical for memory consolidation, in cortical olfactory regions. During anesthesia, a novel odorant mixture activated c-Fos similar to that found in awake animals. Repeated exposure to the odorants caused an attenuation of c-Fos regardless of whether the initial exposure occurred while anesthetized or awake. This suggests that memory-related protein changes can occur in olfactory cortical regions despite anesthesia.

Given, the potential information consolidation observed in these ketamine/xylazine anesthetized animals, we attempted to retrieve olfactory memories using additional behavioral paradigms and memory-enhancing drugs in chapter 3, but were unable to reveal any explicit memory formation. However, we found subtle changes in odorant approach latency, suggesting a potential implicit memory. Further histological investigation in chapter 4 revealed that changes in odor-induced neurogenesis could also occur despite ketamine/xylazine anesthesia, similar to that found with c-Fos labeling, thus supporting the permissive role of this anesthetic. However, odor-related changes in c-Fos did not occur with the anesthetics pentobarbital or propofol, suggesting these traits are not universal amongst all anesthetics. Nevertheless, these data indicate a functional disconnection between the cortical representation of an environmental stimulus and the corresponding perceptual experience or memory.

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PREFACE

I owe much to my mentor, Yan Xu, who has taught me much about science and allowed me to tackle some fascinating questions. He has also provided many valuable lessons about life which aided my progression as a researcher and a person. My entire committee has been very helpful. I owe many thanks to Nathan Urban who was very willing to chair my thesis committee, participate in my comprehensive exam and assist in other areas despite his many other responsibilities. Floh Thiels has been invaluable in providing the knowledge and resources for our behavioral experiments. She was always willing to talk even though I often dropped in at the most inopportune times. I would like to thank Kathy Albers, who personally called me over six years ago to inform me that I was admitted to the neuro program, and who is now seeing that process come full circle. I would also like to thank Gregg Homanics who has taken the time to counsel me on my project and who has also challenged me at the retreat basketball courts. There is truly a great scientific community here in Pittsburgh. I am also grateful to my outside examiner, Keith Miller, for traveling all the way from Boston to share his expertise on anesthesia mechanisms.

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1.0 GENERAL INTRODUCTION

The only proof of there being retention is that recall actually takes place.

–William James (1890)

There may exist a discrepancy between what we know and what our brain 'knows'. That is, our conscious perception may belie the full extent of information that is actually stored in our brains. One striking example of this comes from the blindsight literature. In the mid-1970s it was discovered that individuals, who were perceptually blind due to damage to primary visual cortex, appeared to retain some visual capacity (Weiskrantz et al., 1974). When prompted to guess, blindsight patients are able to strikingly discriminate object orientation, movement and wavelength information though they deny seeing anything (Weiskrantz, 1996; Cowey, 2010). These findings have been shown to extend to individuals with whole cerebral hemispherectomy (Ptito and Leh, 2007), in monkeys with lesions to the primary visual cortex (Cowey and Stoerig, 1995; Stoerig and Cowey, 1997) and even across auditory (Garde and Cowey, 2000) and tactile modalities (Paillard et al., 1983). In the case of blindsight, it is thought that this sub-conscious information may reside in auxiliary visual regions (Leh et al., 2006; Ptito and Leh, 2007). While the exact neuronal correlates of blindsight remain unclear, these studies elucidate a discrepancy between what information is perceived and what information is integrated into the brain.

Many parallels exist between blindsight and the anesthetized brain state. In the anesthetized brain, disrupted sensory processing produces unconsciousness and amnesia of perianesthetic events (Franks, 2008); however, a fair degree of sensory signaling still occurs. Brain imaging and electrophysiological studies have revealed that information can still be transmitted during general anesthesia, and that some information is even received by higher cortical areas where perception is thought to occur (Haberly, 1969; Nemitz and Goldberg, 1983; Onoda et al., 1984; Bonhomme et al., 2001; Veselis et al., 2005; Rinberg et al., 2006). Both blindsight and anesthesia produce a lapse in perception and involve a mix of preserved and disrupted brain functions. Similar to that found in blindsight, it is possible that in the anesthetized brain some level of information may still be registered, and perhaps is even accessible. The potential and extent of information integration during anesthesia and information retrieval following anesthesia are the foci of this dissertation.

1.1 MEMORY AND ANESTHESIA

The extent to which anesthesia affects memory acquisition versus memory retrieval is not well understood. Memory recall is known to be impaired following general anesthesia; however, as William James pointed out over a century ago, failure to recall a memory does not prove that the memory trace does not exist (James, 1890). Since that time, numerous studies investigating the causes of memory failure have revealed much about the nature of memory. In the 1940's, a series of studies showed that amnesia could be induced when electroconvulsive shock (ECS) was applied following complex maze learning in the rat. The severity of the deficit increased the sooner the ECS treatment followed learning (Duncan, 1945, 1948, 1949). It was thought that

this window of efficacy suggested a period of memory lability, termed memory consolidation, where new memories are susceptible to factors that enhance, inhibit or alter the memory trace before it becomes stable (Glickman, 1961; Sara, 2000; Sara and Hars, 2006). Aside from ECS, a number of agents, when administered during this period, produce experimentally-induced amnesia including: protein synthesis inhibitors, ethanol, hypoxia, hypothermia and antagonists of *N*-Methyl-$_D$-aspartic acid (NMDA) receptors, β-adrenergic receptors, polysialylated neural cell adhesion molecule (PSA-NCAM) and cholinergic function (Hayes, 1953; Potts and Bitterman, 1967; Jensen et al., 1975; Rose, 1995b; Przybyslawski et al., 1999; Tronel and Sara, 2003; Carballo-Marquez et al., 2007; Spinetta et al., 2008). Experimentally induced amnesia can also be produced by general anesthesia administered during the period of consolidation (O'Gorman et al., 1998; Alkire and Gorski, 2004; Imre et al., 2006). Thus it is possible that anesthesia-induced amnesia results from a disruption of initial information consolidation processes.

However, others have challenged the idea that memory failure results from an interruption in normal consolidation (Lewis and Maher, 1965; Miller and Springer, 1973). Experimentally induced amnesias were shown to spontaneously reverse or decay over time in some cases (Zinkin and Miller, 1967; Kohlenberg and Trabasso, 1968; Sara and Hars, 2006), whereas in others, re-exposure to the training environment or a learning cue could reverse amnesia and facilitate memory retrieval (Quartermain et al., 1972; Devietti and Hopfer, 1974). Alternatively, amnesias, including anesthesia-induced amnesia, could be reversed by nootropic (cognitive-enhancing) or psychostimulant drugs, thus restoring the memory trace (Quartermain and Leo, 1988; Pang et al., 1993; O'Gorman et al., 1998). The resurfacing of amnestic memories indicates that the memory trace must have been stored initially. This would suggest that

anesthesia-induced memory failure results from a deficit in retrieval, and not consolidation, processes.

However in these cases, anesthesia-induced amnesia was produced by fully anesthetic doses immediately after learning, or sub-anesthetic doses during learning. In both cases the animal was mostly lucid for the training event. For associations occurring at clinically relevant doses, where the individual is unresponsive to the environment, a disruption of initial consolidation may be more likely. Today, it is typically thought that surgical doses of general anesthetics act to block information integration into the brain (Alkire et al., 2008). The disruption of initial consolidation may seem intuitive; however, currently there is little direct evidence to support this.

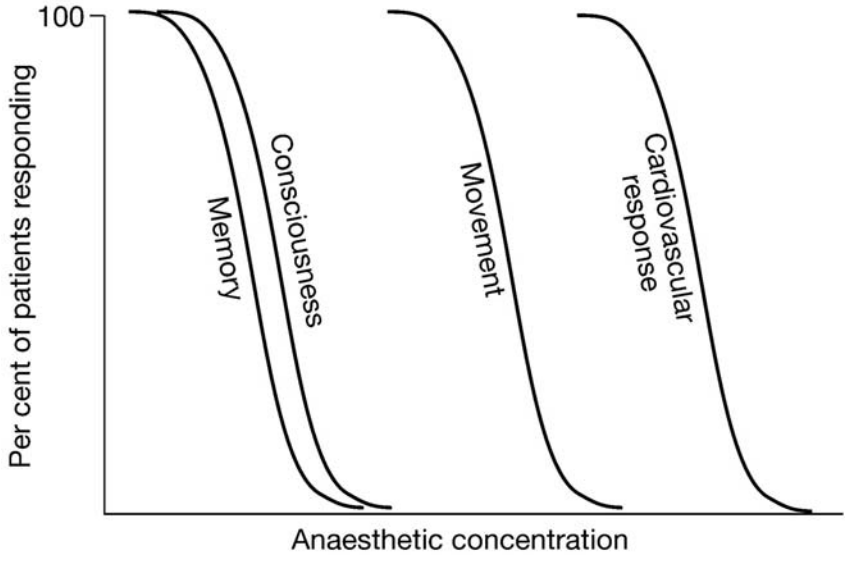

Figure 1-1. Psychological and physiological effects of a typical anesthetic at increasing dose
Image from Antognini and Carstens (2002).

What *is* known about general anesthesia and memory is that these anesthetics can produce robust amnesia of perianesthetic events. General anesthetics produce anterograde amnesia in a dose-dependent manner (Pang et al., 1993; Alkire and Gorski, 2004) and can produce retrograde amnesia at higher doses (O'Gorman et al., 1998). The relative potency of various anesthetics in producing amnesia has been shown to mirror their lipid solubility (Alkire and Gorski, 2004). Memory impairment is thought to occur at lower anesthetic doses (Fig. 1-1) than loss of consciousness (Antognini and Carstens, 2002). Some have suggested that memory is impaired long before consciousness (Alkire et al., 2008). The amnesia produced by anesthetics is typically described in the context of explicit memory. Explicit memory is the conscious recollection of events, facts and people, and is profoundly impaired by general anesthesia (Schwender et al., 1994; Chortkoff et al., 1995; Munte et al., 2000). Interestingly, implicit memory, *i.e.* the unconscious memory behind riding a bike or priming effects, may still remain intact (Ghoneim et al., 1999; Lubke et al., 1999; Iselin-Chaves et al., 2005); however, others have refuted this claim (Rich et al., 1999; Munte et al., 2000; Kerssens et al., 2005).

1.2 MECHANISMS OF GENERAL ANESTHESIA

Based on administration route, general anesthetics are divided into intravenous and inhalational anesthetics. Intravenous anesthetics, such as propofol or ketamine, are typically used for the rapid induction of anesthesia, whereas inhalational anesthetics, such as sevoflurane or nitrous oxide, are often used to maintain the anesthetic state. However, these roles are not absolute. There is tremendous diversity in the molecular structure of various anesthetics ranging from single atoms and simple molecules to much larger halogenated ethers, barbiturates and

benzodiazepines. Despite the similar phenotypes produced by these various agents, their structural diversity has proved challenging for scientists to come up with a unifying theory of anesthetic action.

At the molecular level, considerable research has been done regarding the mechanisms of general anesthetics. At the turn of the twentieth century, Hans Horst Meyer and Charles Ernest Overton independently proposed that the fatty membrane components of neurons were the common targets of general anesthetics based on a strong correlation between anesthetic potency and anesthetic solubility in olive oil (Meyer, 1899; Overton, 1901). However, it has since been found that various anesthetics can inactivate the soluble protein firefly luciferase in a clinically relevant dose-dependent manner (Ueda, 1965; Ueda et al., 1976), suggesting that anesthetics could exert their effects directly on proteins. Challenges to the lipid theory have also come from a discrepancy in observed and expected effects with 'non-immobilizers' (Kandel et al., 1996; Perouansky, 2008) and temperature-based effects on lipids (Franks and Lieb, 1982). However, other findings continue to support a lipid hypothesis, such as the observed cut-off effects with long-chain alcohols (Pringle et al., 1981; Raines et al., 1993). Recently, the ligand-gated ion channel has received a lot of attention as a major site of anesthetic action (Franks and Lieb, 1978; Krasowski and Harrison, 1999). It is thought that the binding of anesthetics causes conformational changes to these integral membrane proteins and may alter the channel dynamics (Tang and Xu, 2002; Cui et al., 2008). Today, the exact molecular mechanisms of general anesthesia are not fully understood; however, we do know that anesthetics typically act to potentiate the function of inhibitory ion channels and/or suppress the function of excitatory channels (Krasowski and Harrison, 1999; Rudolph and Antkowiak, 2004), thus hyperpolarizing

the cells and altering excitability. The relative importance of specific receptor types in mediating anesthetic effects is an area of continuing research.

At the systems level, anesthetics typically do not produce a uniform and global reduction of brain activity. Some anesthetics can even activate the brain (Reinstrup et al., 1994; Duncan et al., 1998; Langsjo et al., 2005). As a result, it has been proposed that general anesthetics act by preferentially affecting critical regions of the central nervous system (Eger et al., 1997). For example, propofol is proposed to produce sedation through effects at frontal and other cortical regions, hypnotic effects (unconsciousness) through the thalamus and midbrain reticular formation and immobility at the level of the spinal cord (Fiset et al., 1999; Alkire et al., 2000; Bonhomme et al., 2001; Rudolph and Antkowiak, 2004; Fiset et al., 2005); however, direct evidence is lacking and considerable variability exists in the brain regions affected by different anesthetics (Veselis et al., 2004).

It may be possible for anesthetics to affect common signaling pathways despite differing molecular targets. One possible pathway is the thalamus as this region is uniquely positioned to gate sensory information to the cortex. Disrupted thalamo-cortical signaling has been implicated in the supraspinal mechanisms of general anesthetics (Alkire et al., 2000; Fiset et al., 2005) including those anesthetics that increase global activity (Reich and Silvay, 1989; Alkire and Miller, 2005). Imaging studies utilizing various anesthetics have shown marked changes in thalamic activity during the transition to the unconscious state similar to that seen when entering deep natural sleep (Bonhomme et al., 2001; Franks, 2008). Similarly, pharmacological inactivation of the thalamus with γ-aminobutyric acid (GABA) agonists can rapidly induce sleep in awake rodents (Miller and Ferrendelli, 1990), whereas intrathalamic injection of nicotine can cause rats to awaken from anesthesia (Alkire et al., 2007). Thus, it is possible that the thalamus

may act as a switch that allows or prevents coherent sensory information from reaching the cortex (Alkire et al., 2000). However, ablation of the thalamus does not prevent cortical activation (Villablanca and Salinas-Zeballos, 1972), suggesting that other regions must also play a role in anesthetic effects.

Brain stem nuclei involved in sleep and arousal have been implicated in anesthetic-induced unconsciousness (Moruzzi and Magoun, 1949; Franks, 2008). These critical midbrain regions have been shown to be suppressed by volatile anesthetics in a dose dependent manner (Ogawa et al., 1992; Keifer et al., 1996). Various cortical areas, such as mesial parietal and posterior cingulate cortices, are also purported to be important (Alkire et al., 2008). Despite much research, a putative locus of anesthetic action in the brain has not been established and thus a distributed effect may be most likely.

Electroencephalogram (EEG) patterns in the human reveal that general anesthetics initially produce a paradoxical excitation characterized by fast, low-amplitude alpha and beta waves typical of waking states (Gugino et al., 2001). As the patient approaches unconsciousness, these give way to slow, high-amplitude delta waves, characteristic of slow-wave sleep, which are thought to be produced by thalamocortical neurons (Alkire et al., 2000). Deep anesthesia produces a stereotypical burst suppression state, with higher doses eventually producing an isoelectric or flat-line EEG, characteristic of brain death (Clark and Rosner, 1973; Alkire, 1998; Brown et al., 2010). This EEG profile is fairly well conserved across anesthetics, but there are exceptions (Maksimow et al., 2006). Anesthetics also disrupt gamma band coherence, which is thought to represent the neural synchrony between regions (John and Prichep, 2005). Loss of inter-region connectivity as a mediator of unconsciousness is also supported by the sleep literature. Giulio Tononi demonstrated that transcranial magnetic

stimulation (TMS) in the cortex produced a cascade of activity that spread throughout connected cortical areas in awake patients. When these patients were again stimulated during slow-wave sleep, the stimulation produced a robust local response; however, there was no spread of activity (Massimini et al., 2005).

In animals anesthetized with steady-state urethane, the EEG state can vary between fast and slow-wave states in the absence of external stimuli (Angel, 1991). In the olfactory system, electrophysiological recordings reveal that olfactory areas are more responsive to odorants when the anesthetized rodent is in the fast-wave, relative to the slow-wave, EEG state (Murakami et al., 2005; Tsuno et al., 2008). In related work, delivery of a memory cue during fast-wave rapid eye movement (REM) sleep was shown to facilitate memory performance, whereas delivery of the cue during slow-wave sleep did not (Hars et al., 1985; Hars and Hennevin, 1987). Clinically, EEG has been shown to have difficulty accurately predicting consciousness; however, the presence of slow-wave EEG appears to correlate well with robust amnesia of explicit memory (Voss and Sleigh, 2007).

It was found that 'non-immobilizers', *i.e.* drugs that fail to produce immobility despite predicted anesthetic qualities, prevented learning and memory without producing complete anesthesia, suggesting that the amnestic effects of anesthesia are mechanistically distinct from other anesthesia endpoints (Kandel et al., 1996; Perouansky, 2008). Some think that anesthetic-induced amnesia may not necessarily be produced through deficits in putative areas of memory storage (*i.e.* hippocampus/medial temporal lobe), but may be produced through effects on distributed cortical areas. In a positron emission tomography (PET) study, propofol-induced amnesia was shown to peak at doses that caused reduced cerebral blood flow in working memory regions, specifically prefrontal and posterior parietal regions (Veselis et al., 2002). However,

virtually all anesthetics have also been shown to have an effect at the hippocampus as well (Wakasugi et al., 1999), and this region has been suggested to play a large role in the amnestic effects of anesthesia (Grimm et al., 1997; Ren et al., 2008; Perouansky and Pearce, 2011). Reduced arousal from brainstem regions could also contribute to anesthetic-induced amnesia (Yasoshima et al., 2006; Shea et al., 2008).

Interestingly, although higher-order cortical areas are thought to be disrupted by anesthesia, primary sensory regions of the cortex appear to retain normal activity during the presentation of sensory stimuli (Rudolph and Antkowiak, 2004; Alkire et al., 2008). Similarly, in vegetative patients environmental stimuli are able to elicit robust brain activity in primary sensory cortices, but not in higher-order regions (Laureys et al., 2002).

1.3 IMMEDIATE-EARLY GENES AND MEMORY

As James (1890) indicated, the fundamental flaw in memory research is that memory acquisition is often inferred based on behavioral recall. If the information was stored, but is unable to be retrieved, analysis of recall alone would obscure this fact. Teasing apart consolidation and recall is a challenge, but is becoming more accessible as imaging techniques and other technologies improve. It is generally believed that initial memory formation results from changes in synaptic efficacy which is strengthened by the synthesis of new proteins. Other competing hypotheses exist; however, most indicate that *de novo* protein synthesis is involved (Alkire and Guzowski, 2008; Hernandez and Abel, 2008; Miyashita et al., 2008). The importance of protein synthesis in memory formation was implicated early on as a number of studies demonstrated that intracranial injection of protein synthesis inhibitors prevented long-term, but not short term memory

formation (Flexner et al., 1963; Agranoff et al., 1965; Potts and Bitterman, 1967). The involvement of new protein synthesis in memory formation suggests that it may be possible to visualize the mechanisms of consolidation through monitoring changes in protein expression.

Immediate-early genes (IEGs) are a class of genes that are rapidly and transiently induced by a variety of stimuli without the need for prior protein expression (Guzowski, 2002). These genes constitute the first wave of transcription in response to a stimulus. In short, patterned synaptic activity increases the intracellular Ca^{2+} content, which activates a series of protein kinases. These kinases, in turn, activate specific transcription factors which induce robust IEG transcription within minutes of the initial stimulation (Greenberg et al., 1986; Guzowski, 2002). Though IEGs can be expressed in the presence of protein synthesis inhibitors, these genes can influence the expression of a large number of proteins (Terleph and Tremere, 2006), thus they are well positioned to mediate the amnestic effects of protein synthesis inhibitors described above. Some IEGs encode "effector" proteins that directly influence neural function; however, the majority of IEGs encode regulatory transcription factors which impact a broad array of targets described as late-response genes (Herdegen and Leah, 1998; Lanahan and Worley, 1998; Tischmeyer and Grimm, 1999). The exact mechanisms by which these activated transcription factors enhance synaptic efficacy and promote consolidation are not well understood. However, in one example, it has been shown that IEGs, such as c-Fos, can activate matrix metalloproteinases (MMPs) which enzymatically act at synaptic structural proteins to induce neural remodeling (Kaczmarek et al., 2002).

Over 40 immediate early genes have been discovered including *c-fos*, *egr-1* (*i.e. zif268 or krox-24*), *c-jun* and *arc*. The IEG, *c-fos*, and its gene product, c-Fos, belong to the leucine-zipper family of transcription factors which includes Fra-1, Fra-2 and FosB (Terleph and Tremere,

2006). Knockdown or knockout of c-Fos impairs long-term memory, but not short-term memory (Paylor et al., 1994; Mileusnic et al., 1996; Grimm et al., 1997; Fleischmann et al., 2003; Yasoshima et al., 2006), a similar pattern to that seen with protein synthesis inhibitors suggesting that c-Fos may be functionally related to protein-dependent memory processes. Knockdown of the related IEG, Arc, produces a similar memory impairment (Guzowski et al., 2000; McIntyre et al., 2005). IEG knockdown prior to the training phase impairs memory, however knockdown prior to the behavioral test has no effect (Lamprecht and Dudai, 1996), indicating that IEGs are involved in memory storage but not retrieval. In general, immediate early genes are elicited by patterns of neural activity that produce long lasting changes in synaptic efficacy, *i.e.* long-term potentiation (LTP) (Abraham et al., 1991; Kaczmarek, 1992; Abraham et al., 1993; Worley et al., 1993), and disruption of IEGs can impair the maintenance of LTP (Guzowski et al., 2000; Fleischmann et al., 2003). Several researchers have proposed that immediate early genes are critically involved in the mechanisms of memory consolidation (Kaczmarek, 1993; Tischmeyer and Grimm, 1999; Guzowski, 2002; Alkire and Guzowski, 2008). This does not imply that immediate early gene expression indicates the presence or absence of a memory trace, but as John Guzowski's group describes (in relation to the immediate early gene, *arc*): "…*Arc* is merely one element in a network of genes that interact in a coordinated fashion to serve memory consolidaton (Miyashita et al., 2008)."

1.4 ORGANIZATION OF THE OLFACTORY SYSTEM

Olfactory information from the environment is detected by olfactory sensory neurons (OSNs) within the olfactory epithelium. The OSNs send information from the epithelium to spherical

dendritic clusters in the main olfactory bulb called glomeruli (Scott et al., 1993). When a particular odorant binds to the olfactory epithelium it activates a discrete set of OSNs expressing the same olfactory receptor, which in turn activates a unique spatial pattern of glomeruli described as an 'odor map' (Uchida et al., 2000; Korsching, 2002; Xu et al., 2003). Different odors each produce a characteristic odor map in the olfactory bulb. The main olfactory bulb is the initial stage of odor processing in the brain (Fig. 1-2) and is composed of three main cellular layers: the glomerular, mitral and granule cell layers. The glomerular and granule cell layers compose the superficial and deepest layers of the bulb, respectively, and contain interneurons that are thought to enhance odor discrimination and sensitivity (Mori et al., 1999; Lledo and Lagier, 2006; Giridhar et al., 2011). The mitral cell layer contains mitral and tufted cells which are the excitatory projection neurons that relay information to downstream targets. The olfactory bulb also receives significant centrifugal inputs, from various brain regions, thought to modify odor arousal and processing (Mouret et al., 2009).

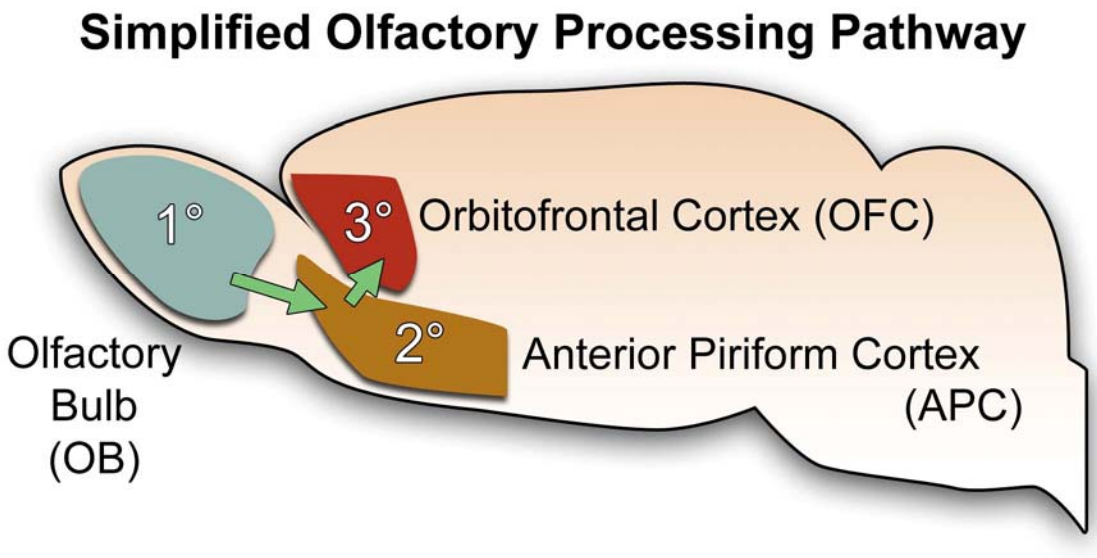

Figure 1-2. Schematic of information flow in the olfactory system

The projection neurons of the olfactory bulb send information via the olfactory tract to the piriform cortex, the largest of the cortical olfactory areas, and other regions including the anterior olfactory nucleus, amygdala and entorhinal cortex (Shepherd, 2007). The piriform cortex is a phylogenetically old, three-layered archicortex and is a critical secondary region of olfactory processing (Fig. 1-2). The odor maps produced in the olfactory bulb are not preserved in the piriform cortex, instead projections from each glomerulus are distributed widely throughout the piriform cortex creating distributed clusters in the anterior piriform cortex (APC) and dispersed connections in the posterior piriform cortex (Luskin and Price, 1982; Haberly, 1985, 2001). Anterior and posterior piriform cortices display unique electrophysiological properties (Jung et al., 1990; Litaudon et al., 1997). The neurons of the anterior piriform cortex show greater c-Fos immunoreactivity in response to olfactory stimulation than those of the posterior piriform cortex (Datiche et al., 2001; Roullet et al., 2004). The APC also receives the greatest amount of input directly from the olfactory bulb and is the primary relay to higher cortical regions (Haberly and Bower, 1989; Illig, 2005), thus we chose to focus on this region of the piriform cortex.

This secondary region of olfactory processing (Fig. 1-2) displays odor receptive fields similar to that found in the olfactory bulb; however, these APC neurons show greater odor specificity (Wilson, 2000a, b) and also exhibit a rapid odor habituation despite sustained bulbar activity (Wilson, 1998). Electrophysiological studies demonstrate that the APC is a mix of associative (responding when odor is paired with reward) and purely sensory neurons (Schoenbaum and Eichenbaum, 1995; Roesch et al., 2007). However, c-Fos immunolabeling in

the APC suggests that these neurons appear to be similarly activated by sensory and associative stimuli (Datiche et al., 2001; Tronel and Sara, 2002; Roullet et al., 2005a).

The APC has been found to be essential for the discrimination of odors (Staubli et al., 1987b; Wilson, 2001; Wilson and Stevenson, 2003) and is thought to be involved in olfactory learning and memory (Barkai and Saar, 2001; Sanchez-Andrade et al., 2005), but not recall (Roullet et al., 2004). Lesions to the piriform cortex significantly impair the ability of rats to acquire new odor discriminations. Effects are greatest for the acquisition of complex odorant combinations relative to simple odorants (Staubli et al., 1987a). In one study, it was found that rats responded to stimulation of the olfactory tract (leading to the piriform cortex) as if it were a specific odorant stimulus (Roman et al., 1987). Rats could then learn to discriminate different types of stimulation (*i.e.* odor surrogates) in a process that produced significant long-term potentiation (LTP) in the piriform cortex (Roman et al., 1987; Litaudon et al., 1997), suggesting that the piriform cortex may be a critical step in the acquisition of olfactory memories.

Information processed in the piriform cortex is projected to the tertiary region of olfactory processing, the orbitofrontal cortex (OFC; Fig. 1-2). The orbitofrontal cortex receives converging multimodal information, yet is the primary region of olfactory processing in the neocortex. The OFC receives olfactory information directly from the piriform cortex and indirectly through the thalamus (Shepherd, 2007). Electrophysiological studies reveal that the OFC encodes a greater degree of associative content than that found in the APC (Roesch et al., 2007) with similar results found through c-Fos immunolabeling (Tronel and Sara, 2002). Lesions to the OFC do not impair odor discrimination or the ability to form simple odor memories; however, a significant impairment is found in higher-order cognitive functions such as overcoming a learned non-reward (Tait and Brown, 2007). In a human fMRI study it was

revealed that banana odor-induced OFC activity was reduced after bananas were consumed to satiety; however, the OFC response to a control odorant (vanilla) remained unchanged after banana consumption (O'Doherty et al., 2000), indicating that the OFC influences the cognitive salience of olfactory information. Taken together, these studies demonstrate a transition in the content of olfactory information as it ascends from the olfactory bulb, up through the anterior piriform cortex to the orbitofrontal cortex. The function of brain activity in these three regions will be the focus of our histological studies.

2.0 IMMUNOHISTOCHEMICAL CONSOLIDATION OF ANESTHESIA-PAIRED STIMULI IN THE ABSENCE OF BEHAVIORAL RECALL

2.1 INTRODUCTION

Two of the defining characteristics of an anesthetic are the ability to induce both a loss of consciousness and amnesia. Prior to the use of diethyl ether as a surgical anesthetic, pioneering anesthesiologists noticed that individuals participating in 'ether frolics' were left with no memory of the event (Fenster, 2001). Since then, general anesthesia has thoroughly been shown to impair or prevent learning and memory formation (Weinberger et al., 1984; el-Zahaby et al., 1994; Rich et al., 1999; Antognini and Carstens, 2002; Alkire and Gorski, 2004; Bekinschtein et al., 2009). Despite the absence of memory, information is still transmitted. Brain imaging in humans has revealed that stimuli presented during deep anesthesia can still reach higher cortical areas, albeit to a lesser degree (Bonhomme et al., 2001; Veselis et al., 2005). Rodent electrophysiological studies in the primary region of olfactory processing (the olfactory bulb) have revealed that anesthesia does not interrupt the neural response to an odorant, but actually improves the signal to noise ratio (Rinberg et al., 2006). Odor responsive neurons were shown to persist during recordings in secondary and tertiary regions of olfactory processing despite anesthesia (Nemitz and Goldberg, 1983; Onoda et al., 1984; Murakami et al., 2005), suggesting

that entire pathways can retain a certain level of function. We were therefore interested whether changes in gene expression would reflect stimuli administered during general anesthesia.

Early studies found that administration of protein-synthesis inhibitors prior to, or shortly after, learning prevented memory formation (Agranoff et al., 1965; Barondes and Cohen, 1966, 1967; Hernandez and Abel, 2008). This work has revealed a window of protein synthesis critical to memory formation termed the 'consolidation' period (Sara, 2000; Sara and Hars, 2006). In order to elucidate molecular events underlying memory consolidation, we investigated the expression of immediate early genes during this period. Immediate early genes (IEGs) are a group of rapidly and transiently activated genes that reflect some level of neural activity (Sheng and Greenberg, 1990; Guzowski, 2002). The transcription factor-encoding IEG, *c-fos*, was examined as this gene has been shown to be critical in memory formation (Paylor et al., 1994; Grimm et al., 1997; Fleischmann et al., 2003). *c-fos* is a proto-oncogene whose gene product, c-Fos, dimerizes with Jun proteins to form the AP-1 (activator protein-1) complex which upregulates a wide variety of genes (Sheng and Greenberg, 1990). The expression of *c-fos* was compared to that of another immediate early gene, *egr1*, which has been previously used as a marker of neural activation (Inaki et al., 2002; Mandairon et al., 2008). In this part of the investigation we studied whether the immunohistochemical changes associated with novel odorant exposure in the awake rat brain occurred similarly to that in the anesthetized animal. We have found that c-Fos expression may reflect the mechanisms of olfactory memory consolidation and that this processes occurs despite presentation of the odorants during the unconscious, anesthetized state.

2.2 MATERIALS AND METHODS

2.2.1 Experimental Animals

For all experiments, 7-week old male Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals were maintained in a controlled temperature (20-22ºC) and humidity environment with a constant 12:12 light-dark cycle (on 07:00 - 19:00 hr). Animals were individually housed to minimize incidental odors and allowed one week to acclimate to the colony and odor environment prior to treatment. The environmental odors in the housing facility were also strictly controlled to minimize any variation. Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

2.2.2 Experiment A: Six day training paradigm

Table 2-1. Group Layout: Experiment 'A'

Group	*Training (days 1-6)*		*Testing (day 7)*		*n* =
A1	AIR	*Awake*	SNT	*Awake*	7
A2	AIR	*Awake*	SNT	Anes	8
A3	SNT	*Awake*	SNT	*Awake*	7
A4	AIR	*Awake*	AIR	Anes	8
A5	Naïve		Naïve		8
					Total = 38

2.2.2.1 Procedure

All procedures were performed inside a hard-ducted flow hood which vented directly to the exterior of the building in order to control incidental odors and preserve odorant novelty. Utmost caution was used to control all odors in the environment, and surfaces were cleaned thoroughly between trials with 70% ethanol then again with water. A clean, empty, typical rat cage (Allentown, Allentown, NJ) was used for the each trial. The lid and wire food grate were removed and replaced with a plate of glass that covered the cage and allowed filming from above. A 1x1" grid was placed beneath the cage to facilitate behavioral scoring. The grid could be seen through the clear plastic caging and was visible to the camera. In experiment 'A', rats were trained for six consecutive days and tested on the seventh day. Training and testing occurred as described in Table 2-1. Experimental animals were either awake (Awake) or anesthetized (Anes) and received either the scent (SNT) or no scent (AIR). A naïve group did not receive any treatments, but was sacrificed immediately after removal from the home cage. All animals received either anesthesia or saline (0.3ml) injections prior to each trial. For ubiquitous bulbar activation, the scent used was an equal mixture of 6 different odorants. These odorants were obtained from Sigma (St. Louis, MO) and included: isoamyl acetate, cineole, octanol, decanal, capronaldehyde, and *R*-(-)-carvone. A total of 38 animals were used in the experiment.

One day prior to training, rats were allowed to acclimate to the novel cage environment for 30 minutes. An empty, capless 1.5 ml Eppendorf (Hauppauge, NY) tube was placed in the center of the cage halfway through to allow the rat to familiarize itself with the object before being returned to its home cage. During training on the following day, rats were placed in a new cage and allowed to acclimate to the environment for 20 minutes with an empty Eppendorf tube.

After 20 minutes, the empty tube was removed, filming was initiated and either a scent-filled or a new empty tube was reintroduced. Awake rats in the 'SNT' condition were given a capless 1.5 ml Eppendorf tube filled with 35 µl of the undiluted odorant mixture injected into a small piece of cotton at the bottom of the tube which acted as a reservoir for the odorants. Tubes filled with the odorant mixture were prepared ahead of time and stored in an air-tight container. Rats in the 'AIR' condition were given a similar tube that did not contain the odorants. Tubes were placed in the opposite corner relative to the rat's location. The animals were allowed to freely interact with the respective tube for 20 minutes, after which the tube and lid were removed and replaced with a wire grate for 15 min to allow the removal of any lingering odors by the flow hood. Anesthetized rats receiving the odorant mixture were placed flat on their stomachs with the odorant-filled tube placed approximately 1-inch from the snout. 10 min into the exposure, the tube was removed and the animal tested for depth of anesthesia, after which the tube was returned to its original location for the duration of the exposure. To provide warmth and prevent hypothermia heating pads were placed under the animal's cage and a lamp was positioned above the animal. Body temperature was monitored with a digital rectal thermometer (VWR, Arlington Heights, IL) and maintained between 36-38ºC through the positioning and intensity of heat sources. Following odorant exposure, the rat was transferred to a new empty cage for 15 minutes to allow the removal of residual odors by the hood. Anesthetized rats in the 'AIR' condition were not given a tube, but were otherwise treated identically. Rats were then returned to their home cage where body temperature was similarly monitored and maintained prior to waking up from anesthesia.

2.2.2.2 Behavioral Analysis

Behavioral videos were manually scored in parallel by blinded observers. The duration of rearing and tube-directed smelling behaviors within 1" of the tube was quantified. The latency of rats to approach the tube was also assessed and capped at a maximum of 30 seconds. Movement was manually scored, again by blinded observers, over the entire video (first 7 minutes of 20 minute exposure period). Movement was assessed by the total number of times the base of the tail (border of fur and furless regions) broke a line in the 1x1" grid positioned underneath the cage. Repetitive, stereotyped back-and-forth line breaks were not counted. Only major ambulatory movements involving two or more consecutive line-breaks were counted.

2.2.3 Experiment B: Single day training (Odor Attenuation Task)

Table 2-2. Group Layout: Experiment 'B'

Group	*Habituation (days 1-3)*	*Training (day 4)*		*Testing (day 5)*		*n =*
B1	*AIR & Awake*	AIR	*Awake*	AIR	*Awake*	6
B2	*AIR & Awake*	AIR	*Awake*	SNT	*Awake*	6
B3	*AIR & Awake*	SNT	*Awake*	SNT	*Awake*	7
B4	*AIR & Awake*	AIR	Anes	SNT	*Awake*	6
B5	*AIR & Awake*	SNT	Anes	SNT	*Awake*	6
						Total = 31

2.2.3.1 Procedure

We developed a novel behavioral task entitled the 'Odor Attenuation Task' for testing odor familiarity related to that described previously (Mandairon et al., 2008). These experiments were conducted in the Rodent Behavioral Animal Core (RBAC) of the University of Pittsburgh. Experiments occurred in the dark with red-light illumination. A custom built 17x17" black

Plexiglas floor was constructed to fit inside an open field activity monitor (model ENV-515, Med Associates, St. Albans, VT) of the same dimensions. A removable black Plexiglas cartridge, 2" in diameter, was positioned in the center of the floor, the profile of which acted as the 'odorant zone'. A 0.8cm hole in the center of the cartridge allowed the rodent indirect access to an odorant-containing tube (1.5ml Eppendorf, Hauppauge, NY) positioned within the cartridge. The cartridge lay flush to the floor such that the animal could only detect the central hole. A clear Plexiglas lid was placed atop the activity monitor to contain the odorants within the chamber and allow for video recording from above. Groups of rats received scent (SNT) or no scent (AIR) while awake (Awake) or anesthetized (Anes) during training and testing days as described in Table 2-2. The scent utilized was the same odorant combination described in experiment 'A'. All animals not receiving intraperitoneal injection of anesthesia were given 0.3ml saline injection 30 min prior to any behavioral work each day. All animals received saline injections on the habituation and test days. A total of 31 animals were used for the experiment.

Rats received a day of handling then three days of habituation in the chambers for twenty minutes each day. Halfway through the habituation, the cartridge was removed, the tube replaced with a new empty tube, and the cartridge was re-inserted. On training and testing days, rats were first acclimated to the chamber with an empty tube in the cartridge for 15 minutes. After which, the cartridge was removed and the tube replaced with either an odorant-containing tube or a similar empty tube. During this experimental phase the rat was allowed to freely explore for a period of 5 minutes. The odorant mixture used during this period was diluted 1:1000 in mineral oil (Sigma-Aldrich, St. Louis, MO). 35 µl of the diluted mixture was injected into a cotton reservoir at the bottom of a 1.5ml Eppendorf tube. A diluted odorant mixture was used for the behavioral test to encourage a prolonged and direct smelling behavior, and to

minimize odor contamination of the training apparatus. Tubes filled with the odorant mixture were prepared ahead of time and stored in an air-tight container. Caution was used to prevent incidental premature exposure to the odorants.

After the 5 minute trial animals were briefly returned to their home cage. They were then transferred to a clean, empty rat cage within a hard-ducted hood in a nearby room. Two tubes of the undiluted odorants, or two empty tubes, were then placed in opposite corners of the cage to prevent an odor gradient. The rat was allowed to freely interact with the tubes for 20 minutes. An undiluted odorant mixture was used in this phase of the experiment to mimic the potent exposure in experiment 'A'. After exposure, rats were aired off in a separate cage for 15 minutes before being returned to their home cages. Several hours prior to receiving anesthesia on the training day, rats in the 'Anes' group first received an additional day of habituation to the behavioral chamber to ensure equal familiarity with the other groups. Later the animals were anesthetized and placed directly in an empty cage within the hard-ducted hood for exposure to the undiluted odorants. These animals received a pulsatile odorant exposure to mimic odor exploration while awake. Rats received 10 seconds of potent exposure (tube 1" from snout) in the beginning, middle and end of the 20 minute period, and received moderate exposure (tube ≥ 5" from snout) for the remainder of the time. No tubes were used for anesthetized rats in the 'AIR' condition, but they were otherwise treated identically. Anesthetized animals were then allowed to air off in the hood for 15 minutes before being transferred back to their home cages. All rats given anesthesia were kept warm through the use of lamps positioned above the cage and heating pads positioned underneath the cage. Techniques established to maintain body temperature between 36-38ºC in experiment 'A' were again utilized in experiment 'B'; however, direct measure of body temperature was not assessed.

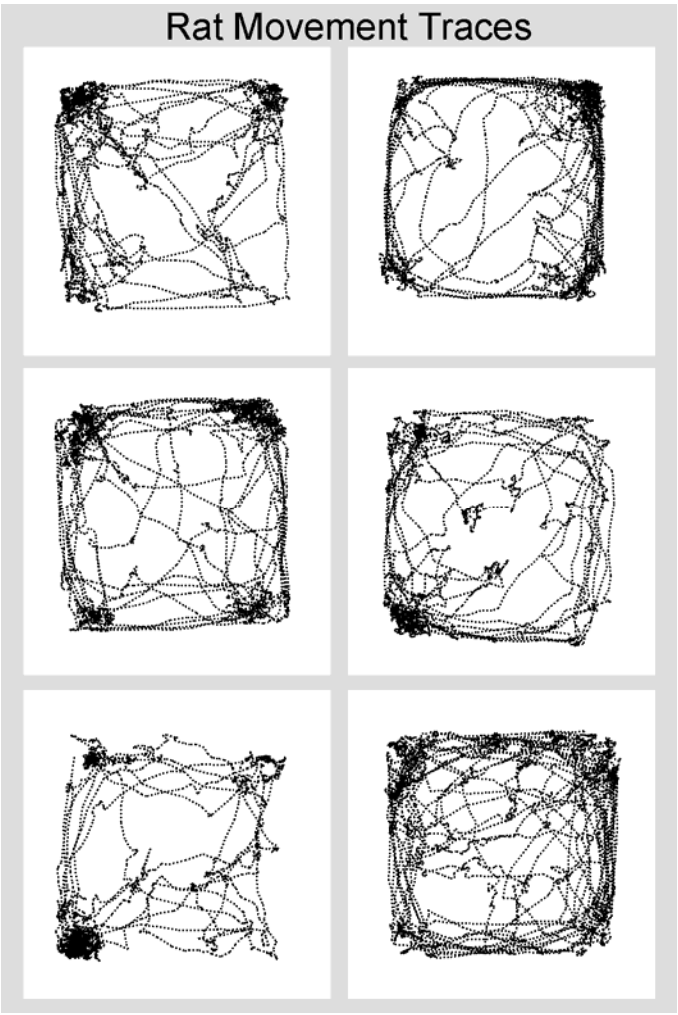

Figure 2-1. Representative path traces demonstrating avoidance of the central chamber region.
Ambulatory traces of the rat body over the 15 minute habituation period just prior to familiarity testing. The majority of time was spent in the corners or along the walls of the chamber; however, most animals appeared willing to enter or cross the central region.

2.2.3.2 Behavioral Analysis

Video was acquired and analyzed using Limelight automated tracking software (v3.0; Actimetrics, Wilmette, IL) with black and white CCD cameras (Panasonic, Secaucus, NJ). Nose, body and tail locations were tracked using the software, and time of the nose within the pre-defined odorant zone was quantified automatically for the first 10, 30 and 100 seconds from

approach. To confirm automated analysis, videos were manually scored for smelling behaviors by two blinded observers (see Appendix A, Fig. A-1). The latency of rats to approach the odorant zone was assessed and capped at a maximum of 30 seconds. All animals in this experiment approached the odorant zone within the time allowed for the trial (5 minutes). Pilot studies found that rats typically avoided the central and most exposed region of the chamber when the experimental odorants were not present (data from habituation phase; Fig. 2-1).

2.2.4 Anesthesia

To ensure a uniform physiological state, rats were deeply anesthetized until they reached a surgical depth of anesthesia. 'Surgical depth' was defined as decreased respiration rate and depth, decreased muscle tone and absence of response to a noxious (repetitive toe pinch) stimulus. Skin color was also used as an indirect measure of blood oxygenation. The anesthetic used was intraperitoneal injection of ketamine (Ketaset, Fort Dodge, Fort Dodge, IA; 100mg/ml) and xylazine (Anased, Lloyd Laboratories, Shenandoah, IA; 100mg/ml). An initial dose of anesthesia was used to immobilize the animals and subsequent booster injections were given until the appropriate plane of anesthesia was reached. Rats were given an average dose of 148.0 ± 13.7 mg/kg ketamine and 19.7 ± 1.8 mg/kg xylazine over an approximate 30 minute period in experiment 'A' and 162.6 ± 10.7 mg/kg ketamine and 21.8 ± 1.4 mg/kg xylazine in experiment 'B'. No significant differences were found between group averages for anesthetic dose administered in either experiment. Doses of ketamine and xylazine (± Standard Error, S.E.) by group for each trial in experiments A and B are represented in Table 2-3.

Table 2-3 Average dose of ketamine/xylazine anesthesia (mg/kg ketamine, xylazine ± S.E.).

Group	*Train*	*TEST*
A2		125.1 ±13.5, 16.7 ±1.7
A4		170.8 ±22.4, 22.8 ±3.0
B4	159.7 ±19.0, 21.4 ±2.5	
B5	165.6 ±11.6, 22.2 ±1.6	

2.2.5 Immunohistochemistry

Two hours after odorant/air exposure on the testing day, animals were sacrificed with anesthetic overdose and perfused with a solution of 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.0). Brains were post-fixed overnight and cryoprotected in a 30% sucrose/PBS solution with 0.01% sodium azide as a preservative. 50µm sections (experiment A) and 30µm sections (experiment B) were generated using a sliding microtome (Leica, Buffalo Grove, IL) equipped with a freezing stage (Physitemp Instruments, Clifton, NJ). Tissue was prepared for either brightfield or fluorescent microscopy. Sections were blocked with 10% donkey blocking buffer (PBS with 10% donkey serum and 0.2% Triton X-100) for 2 hours at room temperature. Primary antibodies against c-Fos (Calbiochem-EMD Biosciences, San Diego, CA; 1:5000), Egr1 (Cell Signaling, Boston, MA; 1:10,000), NeuN (Chemicon-Millipore, Billerica, MA) and/or GAD67 (Abcam, Cambridge, MA) were diluted in donkey blocking buffer and incubated with free-floating sections overnight at 4ºC.

Sections for brightfield microscopy were then washed with PBS and incubated in a biotinylated goat secondary antibody (1:1000 in DBB; Dako, Carpinteria, CA) for 2h at room temperature, followed by an avidin-peroxidase wash (1:1000 in DBB; Sigma, St. Louis, MO) for 1h. Enzymatic reaction with DAB (3,3'-Diaminobenzidine; Sigma) was performed until sections

reached optimal darkness (1-3 minutes). Sections were then dehydrated in a series of ethanol and xylene solutions, mounted on glass slides and cover slipped using Eukitt mounting medium (Electron Microscopy Sciences; Hatfield, PA).

Sections for fluorescent microscopy, after treatment with primary antibodies, were washed in PBS and incubated with Alexa Fluor 594 and/or 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA; 1:500) in the dark. Sections were co-stained with the nuclear marker DAPI (4',6-diamidino-2-phenylindole; Sigma), washed and mounted onto slides using fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL).

2.2.6 Quantification

Staining was imaged using an Olympus IX-81 widefield microscope equipped with a color Retiga 2000R (QImaging, Surrey, BC, Canada) and monochrome ORCA-ER (Hamamatsu, Bridgewater, NJ) peltier-cooled CCD cameras. Images were acquired and analyzed using Image-Pro Plus (Media Cybernetics, Bethesda, MD) software. For DAB-labeled sections the optical fractionator method (King et al., 2002) was used to quantify each region. Every sixth section was quantified, using this stereology technique, in the anterior piriform cortex and lateral orbitofrontal cortex (from bregma +3.1mm to +3.7mm, rostrocaudal) (Paxinos and Watson, 1998; Swanson, 1998). In the olfactory bulb, sections at +6.8mm and +7.9mm rostrocaudal bregma were quantified and used to estimate the positive cell number in the bulb. Positive labeling in DAB sections was represented as total or average number of positive cells. Fluorescent images were acquired under identical settings and uniformly quantified by a threshold method. They were additionally screened by a set size and subjected to a *watershed* split function. Comparisons were made only between sections stained and imaged in the same

session. Sections for fluorescent c-Fos and Egr1 imaging were taken from +3.5mm, +3.3mm and +3.1mm rostrocaudal bregma in the cortex. Positive labeling in fluorescent tissue was represented as positive cell number per mm^2. Double-labeling c-Fos/NeuN and c-Fos/GAD67 experiments utilized sections from +3.3mm rostrocaudal bregma in the cortex. Labeling of c-Fos/NeuN was quantified using the threshold method; however, c-Fos/GAD67 double-labeled sections had to be manually counted (in a blinded manner) due to the non-nuclear nature of the GAD67 antibody labeling.

2.2.7 Statistical Analysis

One-way ANOVA was used to determine a significant overall difference followed by Tukey's *post hoc* analysis for inter-group comparison. For other analyses, two-way ANOVA was used followed by a Bonferroni posttest. A student's t-test was utilized for experiments containing only two groups. Values are described as group means ± standard error. For all analyses, differences were considered significant when $p < 0.05$.

2.3 RESULTS

2.3.1 Absence of memory recall for anesthesia-paired odorants

To confirm the effects of anesthesia on memory for our novel odor attenuation task, rats were fully anesthetized and presented a novel odorant mixture during training. These rats were then tested for familiarity with the odorants 24 hours later during the test trial (Fig. 2-2). One-way

ANOVA of odor investigation times (first 30 seconds after approach) for all cohorts in experiment 'B' produced a significant group effect ($F_{(4,25)}$ = 9.039; $p < 0.001$) during the automatically scored TEST trial (Fig. 2-2B). Tukey *post-hoc* test appeared to reveal two distinct behavioral phenotypes. Repeated exposure to the same odorant mixture (SNT-SNT, group B3) produced a significantly attenuated behavioral response relative to a single exposure (AIR-SNT, B2; tukey posttest, $p < 0.05$). Thus, rats previously exposed to the odorants appeared less interested in the odor stimulus (familiar response) relative to rats that experienced the odorants for the first time (novelty response). Importantly, rats that received the odorant mixture and anesthesia on the training day, then the odorant mixture while awake on the testing day (SNT(Anes)-SNT, B5) were significantly different than rats that were tested with air (AIR-AIR, B1; $p \leq 0.01$, tukey posttest) or familiar odorants (SNT-SNT, B3; $p \leq 0.01$, tukey posttest). These animals (SNT(Anes)-SNT, B5) were not significantly different than rats that received the novel odorant mixture after training with air (AIR-SNT, B2; $p = 0.998$) or after training with air and anesthesia (group AIR(Anes)-SNT, B4; $p = 1.00$). Blinded manual scoring (Fig. 2-2C) confirmed these data produced by the tracking software. Likewise, a similar pattern was found when behavioral data were assessed at different time periods (first 10s or 100s after approach), which could also be visualized through representative path traces (see Appendix A). These findings suggest that rats are unable to retrieve the memory of an odorant if the odorant was presented while under general anesthesia.

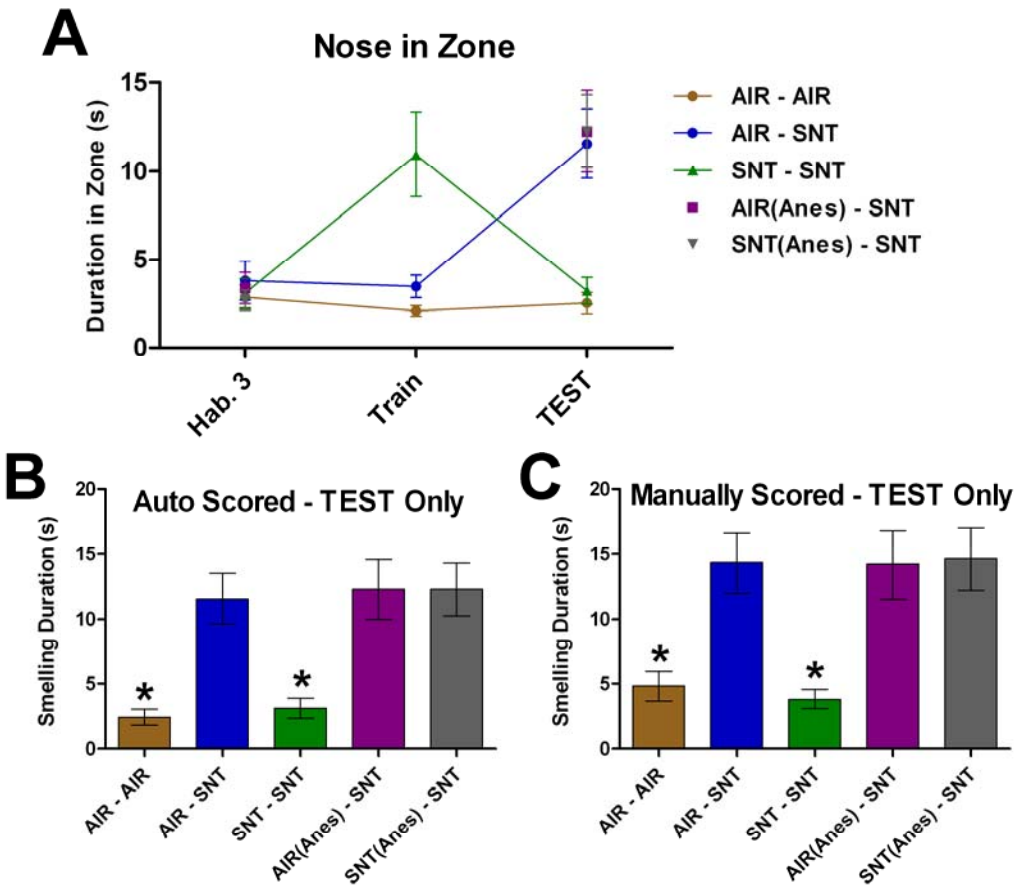

Figure 2-2. Absence of memory for odorants experienced during general anesthesia.

Behavioral familiarity with the odorant mixture was assessed by duration of odor investigation time. (A) Time course of odor response on the final habituation, training and testing trials. Test trial data from (A) is replotted in bar graph form in (B). (B) Smelling duration was determined by the duration of time the snout was within the odorant zone scored automatically within the first 30 seconds after approach. (C) Manual confirmation of automatically scored data by blinded observers. Data from experiment 'B'. (*) $p < 0.05$ relative to AIR-SNT, one-way ANOVA with Tukey *post hoc* test. 'Hab. 3' = Habituation day 3.

2.3.2 Immediate-early gene product, c-Fos, reflects consolidation

In attempt to address the mechanisms of consolidation, we first looked at the pattern of IEG expression in the olfactory cortical regions of awake, odorant-exposed rats. Protein expression was assessed in the primary (Olfactory Bulb, OB), secondary (Anterior Piriform Cortex, APC) and tertiary (Orbitofrontal Cortex, OFC) regions of olfactory processing (Shepherd, 2007) experiment 'A' (Fig. 2-3). Immediate early genes were selected as these genes have been suggested to play a role in memory consolidation (Rose, 1991). Specifically, we wanted to investigate any potential differences between the IEG response during the period of initial consolidation (following a novel odor, group A1) and the post-consolidation period (following a familiar odor, group A3). Naïve animals (A5) were included in figure 2-3 as a negative control. One-way ANOVAs of all groups in experiment 'A' were performed on each brain region for the immediate early gene products, c-Fos and Egr1. Significant group effects were found for both c-Fos and Egr1 in each brain region (see Table 2-4 for specific *F* and *p* values).

Table 2-4. One-way ANOVA *F* and *p* values for all regions and antibodies

c-Fos				Egr1			
OB-GCL	OB-ML	APC	OFC	OB-GCL	OB-ML	APC	OFC
$F_{(4,33)} = 18.00$	$F_{(4,33)} = 31.24$	$F_{(4,33)} = 11.84$	$F_{(4,33)} = 10.64$	$F_{(4,33)} = 8.15$	$F_{(4,33)} = 10.26$	$F_{(4,33)} = 16.14$	$F_{(4,33)} = 28.36$
$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$	$p < 0.001$	$p < 0.0001$	$p < 0.0001$	$p < 0.0001$

Tukey *post-hoc* comparison of c-Fos labeling revealed a significant effect between rats receiving novel (AIR-SNT, A1) and familiar odorant mixtures (SNT-SNT, A3) in the granule cell (OB-GCL) and mitral cell layers (OB-ML) of the olfactory bulb ($p < 0.001$ and $p \leq 0.05$, respectively) but not in orbitofrontal cortex ($p = 0.86$; Fig. 2-3A). A trend was found in the anterior piriform

cortex ($p = 0.10$), suggesting a potential effect in this region as well. No significant differences were found between novel (AIR-SNT, A1) and familiar (SNT-SNT, A3) odorant mixtures for Egr1 expression in any of the brain regions assessed (Fig. 2-3B). However, naïve (no odor, A5) rats demonstrated significantly less Egr1 expression than both novel and familiar odor groups (A1 and A3) in all regions ($p < 0.05$ for each pair-wise comparison). The preferential expression of c-Fos for novel stimuli suggests that this gene is activated during times of memory formation and thus may reflect some level of information consolidation in olfactory-related cortical regions. Egr1, on the other hand, appears to indicate the presence of absence of a stimulus.

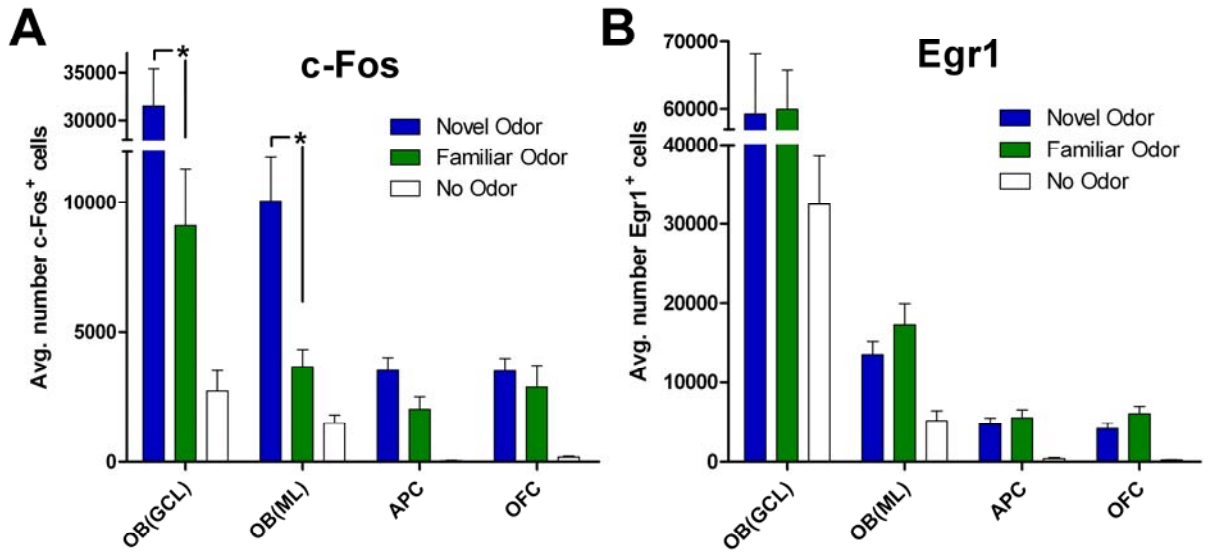

Figure 2-3. Preferential expression of c-Fos during periods of consolidation.

Differential c-Fos (A) and Egr1 (B) expression following novel (AIR-SNT), familiar (SNT-SNT) or no (naïve) odor in the primary [OB(GCL) & OB(ML)], secondary (APC) and tertiary (OFC) regions of olfactory processing. Data from experiment 'A'. (*) $p < 0.05$ between novel and familiar odors, one-way ANOVA with Tukey *post hoc* test. GCL = Granule Cell Layer of olfactory bulb; ML = Mitral Layer of olfactory bulb.

2.3.3 Time-course of c-Fos mirrors behavior

Previous studies have found that expression of immediate early genes, including *c-fos*, were attenuated following a week of repeated odorant exposure, for twenty minutes each day, relative to a single exposure (Montag-Sallaz and Buonviso, 2002) a finding that we have confirmed in a number of olfactory processing regions (Fig. 2-3A). These studies may suggest that one week of repeated odorant is required to produce c-Fos attenuation. However, comparison of behavioral data for novel (AIR-SNT, A1) and familiar (SNT-SNT, A3) odorant exposures revealed that odor familiarity is acquired on a shorter time-scale. Two-way ANOVA of behavioral data from animals with six days of training (Experiment A) revealed a significant effect of treatment day and the interaction of date and condition ($p < 0.0001$ for both). Bonferonni *post hoc* test revealed a significant effect between animals in the novel (AIR-SNT, A1) and familiar (SNT-SNT, A3) odor groups on training day 1 ($p < 0.001$) and on the test day ($p < 0.001$). Interestingly, by training day 2, animals receiving a familiar odorant mixture (SNT-SNT; A3) were not significantly different than those receiving only air (AIR-SNT, A1). In short, the rodents appeared to be behaviorally familiar with the odorants after only one day, as opposed to one week, of exposure.

If c-Fos expression were to reflect some level of memory consolidation, we might expect to see these histological changes occur on the same time-scale as the behavior changes, which require merely a single exposure. In experiment 'B', rats received only a single day of the odorant mixture (or air) prior to testing. Comparison of behavioral data from experiment 'A' (Fig. 2-4, top) and histological data from experiment 'B' (Fig. 2-4, red arrow) allows us to determine whether c-Fos expression reflects behavioral measures. If c-Fos is attenuated after only a single prior exposure, this would suggest that the temporal expression profile of c-Fos

protein is appropriate for that of a consolidation marker. Analysis of c-Fos histological data (one-way ANOVA) from experiment 'B' indeed reveals a significant group effect in the APC and OFC ($F_{(4,26)} = 5.98$, $p < 0.01$ and $F_{(4,26)} = 6.96$, $p < 0.001$, respectively). Inter-group comparison (tukey posttest) reveals a difference between novel (AIR-SNT, B2) and familiar (SNT-SNT, B3) odorant exposures already by day 2 for the APC ($p < 0.05$) and OFC ($p < 0.01$). Furthermore, the c-Fos attenuation with familiar odorants appears more robust after a single exposure than following seven days of repeated exposure, especially in the OFC (Fig. 2-4, grey arrow). On day 2, the average expression of c-Fos with a familiar odorant was attenuated to 54.5% of a novel odorant in the APC and 29.6% of a novel odorant in the OFC. On day 7, expression was attenuated to 57.5% in the APC and only 82.3% in the OFC. These data suggest that memory formation and changes in c-Fos histology occur in a similar temporal manner. In addition to changes in expression level (Fig. 2-3), the IEG, c-Fos, is also expressed in the appropriate temporal manner as a marker of memory consolidation.

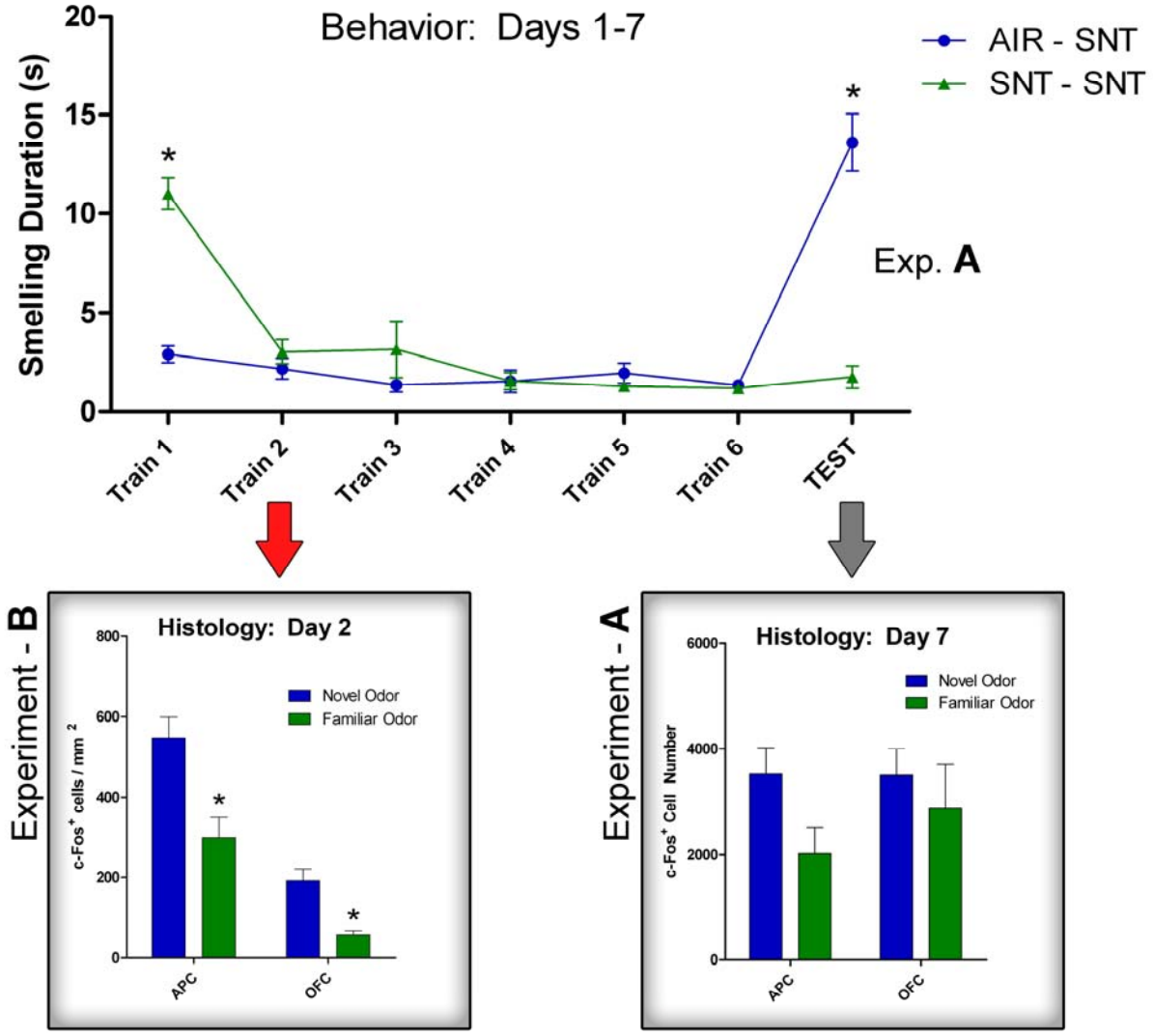

Figure 2-4. Expression of c-Fos matches time-course of memory acquisition.

(Top) Rats receiving 7 days of repeated odorant (SNT-SNT or familiar odor) in experiment 'A' displayed behavioral familiarity with the odorant by training day 2. Corresponding histology of these animals (bottom, grey arrow) revealed significantly attenuated c-Fos in the APC after 7 days of repeated odorant. However, in experiment 'B' (bottom, red arrow) we found that c-Fos expression was attenuated in the APC and OFC after only 2 days of repeated odorant, a time-point that matches behavioral habituation to the odor in experiment 'A'. Data from experiments 'A' & 'B'. (*) $p < 0.05$, two-way ANOVA with bonferonni *post hoc* test, top; one-way ANOVA with tukey posttest, bottom. Note: different quantification techniques used for experiments 'A' and 'B'.

2.3.4 Possible consolidation during general anesthesia

Given the expression profiles of c-Fos and Egr1 in the awake, odor-exposed rat (Fig 2-3), we assessed the pattern of these IEGs when the odorant mixture was presented in the anesthetized state (experiment 'A'). Rats receiving anesthesia and a novel odorant mixture (AIR-SNT(Anes), group A2) were represented alongside those receiving anesthesia alone (AIR-AIR(Anes), A4) as well as positive (novel odor; AIR-SNT, A1) and negative controls (naïve animals, A5; Fig. 2-5). One-way ANOVA of all groups in experiment 'A' revealed a significant group effect in the anterior piriform cortex (secondary region of olfactory processing) for both c-Fos ($F_{(4,33)} = 11.84$; $p < 0.0001$) and Egr1 protein ($F_{(4,33)} = 16.14$; $p < 0.0001$). Interestingly, Tukey post-hoc test revealed a significant difference ($p < 0.05$) in c-Fos labeling between rats that received a novel odorant mixture during anesthesia (AIR-SNT(Anes), A2) and rats that received anesthesia alone (AIR-AIR(Anes), A4). No differences were found between rats who received the odorants while anesthetized (AIR-SNT(Anes), A2) and rats that received the odorants while awake (AIR-SNT group, A1; $p = 0.82$). Egr1 expression did not differ between animals who received a novel odorant mixture during anesthesia (AIR-SNT(Anes), A2) and those who received anesthesia alone (AIR-AIR(Anes), A4).

Our findings also revealed significant group effects in the orbitofrontal cortex (tertiary region of olfactory processing) for both c-Fos ($F_{(4,33)} = 10.64$; $p < 0.0001$) and Egr1 ($F_{(4,33)} = 28.36$; $p < 0.0001$; Fig. 2-6) as shown by one-way ANOVA. Inter-group comparison did not reveal a significant effect of c-Fos labeling between anesthetized rats who received the odorants (AIR-SNT(Anes), A2) and those that did not (AIR-AIR(Anes), A4; $p = 0.30$). In this case, a trend was observed between rats who received the odorants during anesthesia (AIR-SNT(Anes), A2) and rats who received the odorants during the awake state (AIR-SNT, A1; $p = 0.065$).

Again, Egr1 expression did not differ between those who received the novel odorant mixture during anesthesia and those that received anesthesia alone (groups A2 and A4).

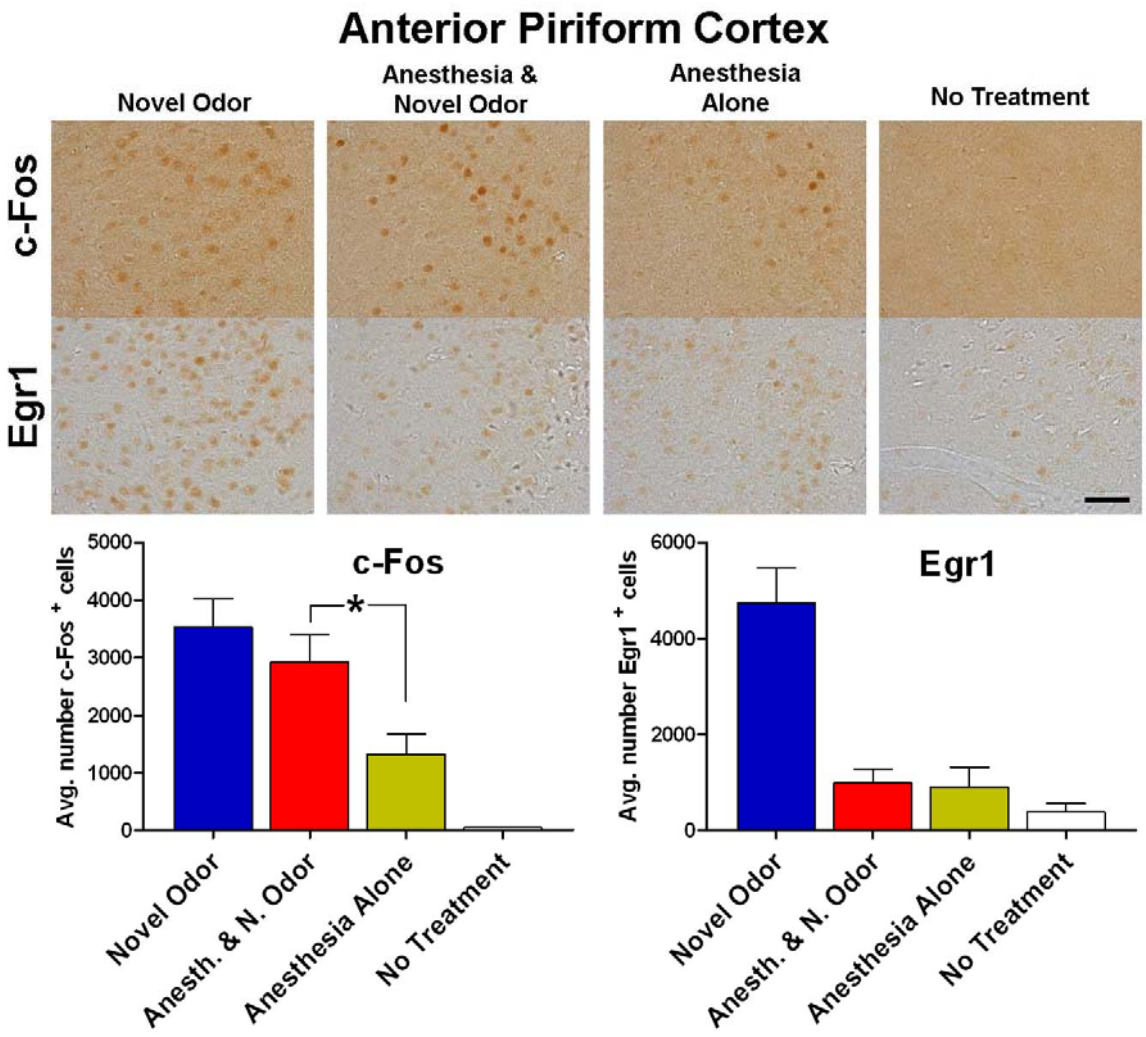

Figure 2-5. Elevated c-Fos elicited by olfactory stimuli during anesthesia in the APC.

Representative photomicrographs (top) of c-Fos and Egr1 immunolabeling in the anterior piriform cortex. Corresponding c-Fos (bottom-left) and Egr1 (bottom-right) stereological quantification. Data from experiment 'A'. (*) $p < 0.05$, one-way ANOVA with Tukey *post hoc* test. Scale bar = 50µm.

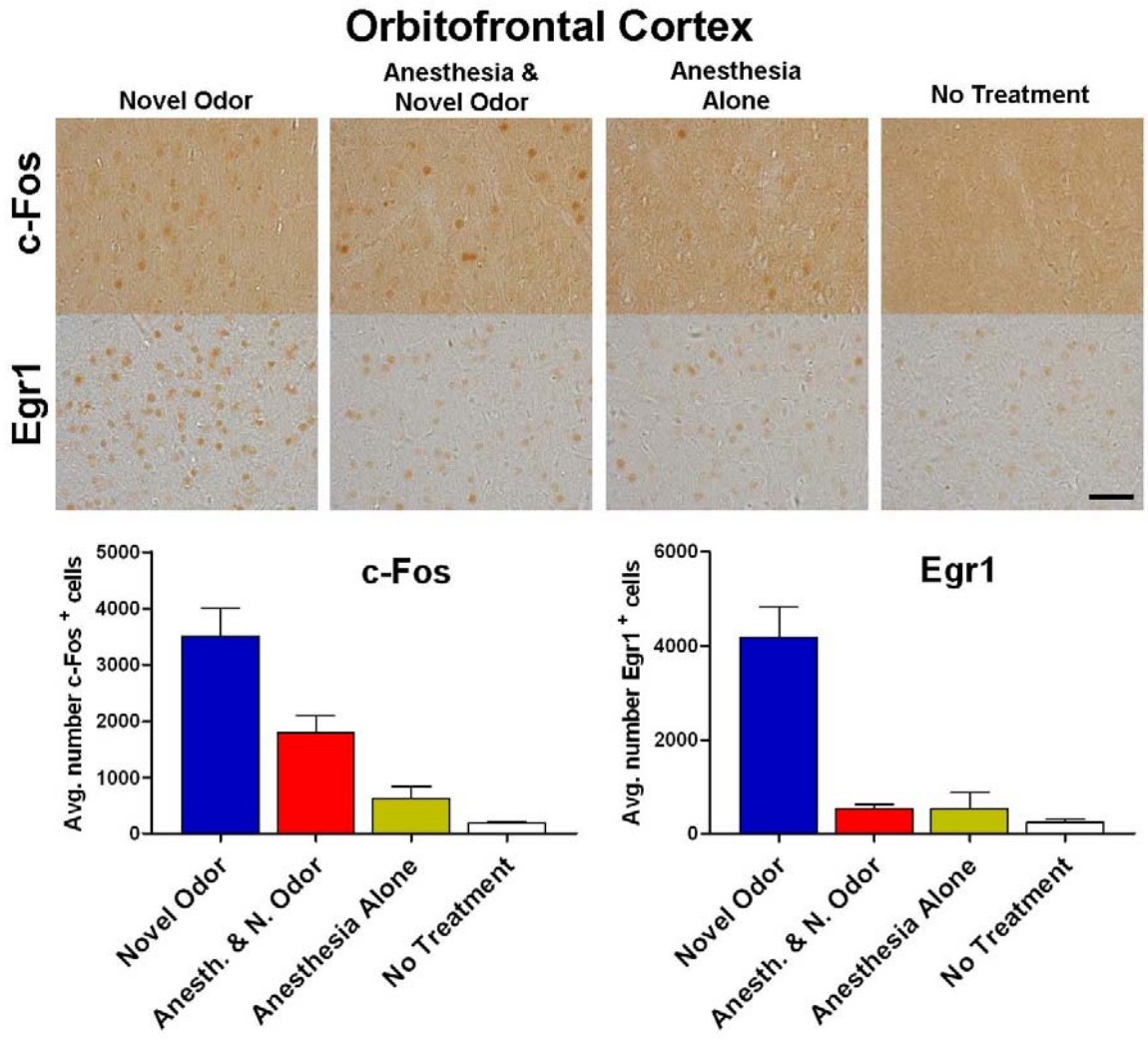

Figure 2-6. Elevated c-Fos elicited by olfactory stimuli during anesthesia in the OFC.

Representative photomicrographs (top) of c-Fos and Egr1 immunolabeling in the orbitofrontal cortex. Corresponding c-Fos (bottom-left) and Egr1 (bottom-right) stereological quantification. Data from experiment 'A'. Scale bar = 50µm.

No significant differences were seen in olfactory bulb c-Fos labeling between anesthetized animals with (AIR-SNT(Anes), A2) and without (AIR-AIR(Anes), A4) exposure to the odorant mixture in either the granule cell layer or mitral layer (Fig. 2-7). These data suggest that some level of information consolidation can occur when olfactory stimuli are presented in the deeply anesthetized state. These changes occurred in the secondary region of olfactory processing (APC) but not in the primary or tertiary regions of olfactory processing (OB and OFC, respectively).

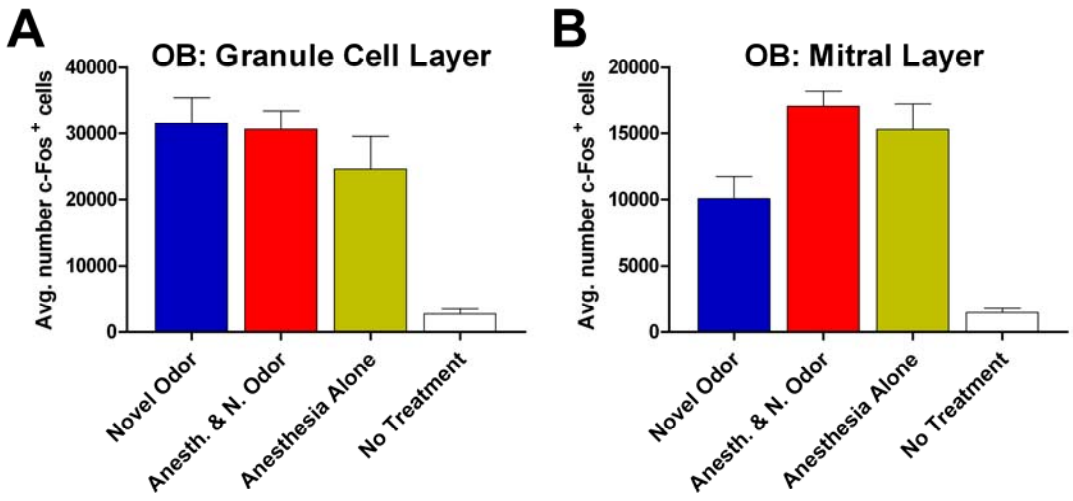

Figure 2-7. No evidence of consolidation in the olfactory bulb.

Quantification of c-Fos protein expression in the granule cell layer (A) and mitral layer (B) of the rat olfactory bulb. Data from experiment 'A'. OB = Olfactory Bulb

2.3.5 Traces of consolidation following general anesthesia

If olfactory stimuli caused c-Fos activation under general anesthesia, it is possible that long-term changes took place to reflect that information. To determine if this olfactory information was stored, we investigated whether stimuli received under general anesthesia would later be reported as novel or familiar through c-Fos histology. Histological data in experiment 'B' is presented juxtapose to behavioral data (Fig. 2-8A, B) for the same animals. Rats who received a novel odorant mixture while anesthetized during training then received the same odorants again while awake on the test day (SNT(Anes)-SNT, group B5) were compared with rats who received anesthesia alone during training and the novel odorant mixture during testing (AIR(Anes)-SNT, B4), along with novel (AIR-SNT, B2), familiar (SNT-SNT, B3), and no odorant (AIR-AIR, B1) groups. One-way ANOVA of c-Fos labeling revealed a significant group effect in the anterior piriform ($F_{(4,26)} = 5.98$; $p < 0.01$) and orbitofrontal cortices ($F_{(4,26)} = 6.96$; $p < 0.001$; Fig 2-8). *Post-hoc* analysis revealed a significant difference between novel (AIR-SNT, B2) and familiar (SNT-SNT, B3) odor groups in the APC ($p < 0.05$; Fig. 2-8C) and OFC ($p < 0.01$; Fig. 2-8D), a pattern that is reflected in the behavioral data (Fig. 2-2B)**.**

Interestingly, the SNT(Anes)-SNT group (B5) was significantly attenuated relative to the novel odor group (AIR-SNT, B2) in both the APC and OFC ($p < 0.01$ for both). Unlike the SNT(Anes)-SNT (B5) group, AIR(Anes)-SNT (B4) animals did not differ significantly from the animals receiving novel odorants (AIR-SNT, B2) in either the APC or OFC. Furthermore, in the orbitofrontal cortex the SNT(Anes)-SNT (B5) group was significantly different than the AIR(Anes)-SNT group (B4; $p < 0.05$; Fig. 2-8D). In these regions, the pattern of c-Fos expression for novel and familiar odorants appeared similar regardless of prior anesthesia exposure, a pattern dissimilar to that found behaviorally. These findings indicate that an odorant

mixture presented during ketamine/xylazine anesthesia produces a histological imprint capable of impacting subsequent gene expression. Egr1 immunolabeling in these two regions did not produce any significant effects.

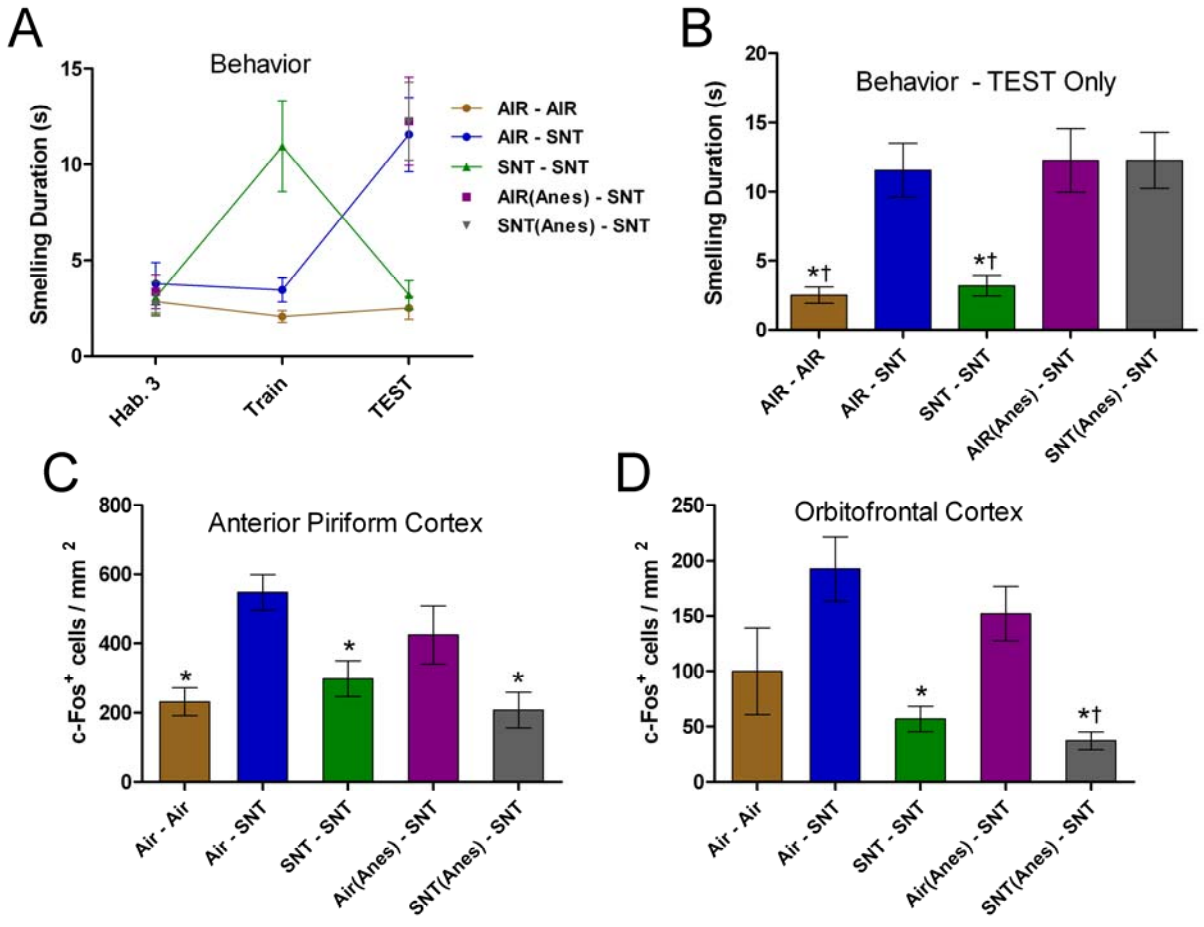

Figure 2-8. Evidence of histological memory trace for anesthesia-paired stimuli – comparison with behavior. Behavioral familiarity with the odorant stimulus on the test trial (A,B) and quantification of c-Fos protein expression in the anterior piriform (C) and orbitofrontal (D) cortices following the test trial. Test trial only data from (A) replotted in bar graph form in (B). Data from experiment 'B'. (*) $p < 0.05$ relative to AIR-SNT; (†) $p < 0.05$ relative to AIR(Anes)-SNT, one-way ANOVA with Tukey *post hoc* test. 'Hab. 3' = Habituation day 3.

2.3.6 Differences between Ketamine/Xylazine and Odor-induced c-Fos

Knowing that both the odorant mixture and ketamine/xylazine anesthesia were able to induce c-Fos expression, we were interested to see whether different sub-populations of cells were activated by these different stimuli. To ensure that the c-Fos-positive cells were indeed neurons, we double-labeled sections from rats receiving the odorant mixture alone (AIR-SNT, A1), the odorant mixture and anesthesia (AIR-SNT(Anes), A2) or anesthesia alone (AIR-AIR(Anes), A4) from experiment 'A' with antibodies against c-Fos and NeuN, a marker of mature neurons. We found that the vast majority of c-Fos-labeled cells were also mature neurons (Fig. 2-9). Across all groups, the average percent of c-Fos-positive cells that were also positive for NeuN was 95.5% ± 1.3 in the APC and 88.3% ± 5.1 in the OFC. One-way ANOVA of these groups revealed a significant group effect in the anterior piriform cortex ($F_{(2,6)} = 5.28$; $p < 0.05$). Tukey *post-hoc* test revealed a significant difference between rats who received a novel odorant mixture (AIR-SNT, A1) and rats who received anesthesia alone (AIR-AIR(Anes), A5; $p < 0.05$). No significant effects were found in the orbitofrontal cortex.

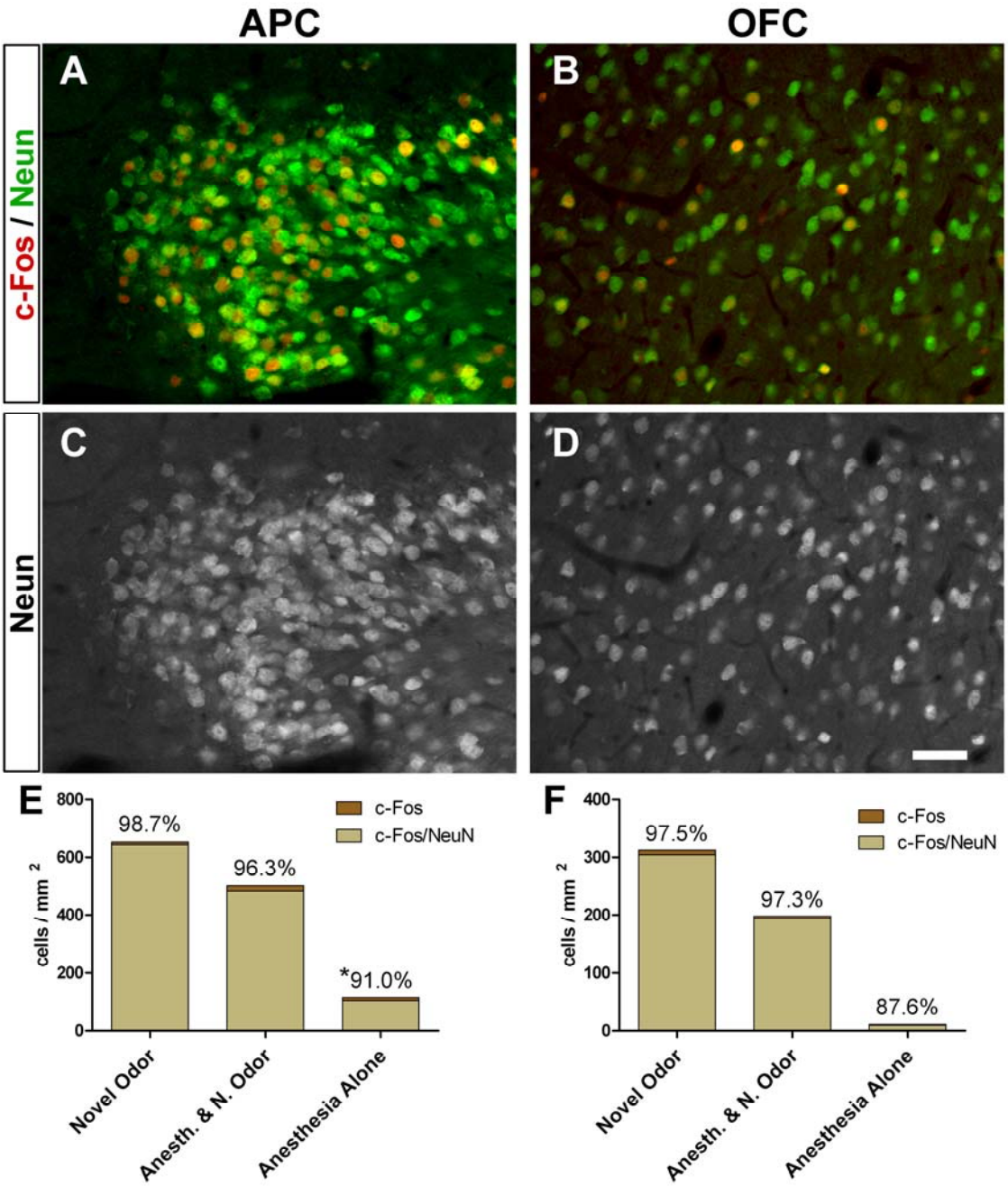

Figure 2-9. Overlay of c-Fos and NeuN expression in the APC and OFC.

Representative fluorescent micrographs of c-Fos (red) and NeuN (green) double-labeling in the APC (A) and OFC (B). Corresponding monochrome NeuN images are represented in (C) and (D), respectively. Quantification of double-labeled c-Fos/NeuN$^+$ cells is represented for the APC (E) and OFC (F). The height of each bar indicates the total density of c-Fos$^+$ cells. The percentage indicated over each bar represents the density of double-labeled c-Fos/NeuN$^+$ cells relative to the total density of c-Fos$^+$ cells. Data from experiment 'A'. (*) $p < 0.05$ relative to Novel Odor, one-way ANOVA with Tukey *post hoc* test. Scale bar = 50µm.

Given that both the odorant mixture and anesthesia alone activate c-Fos largely in neurons, we next addressed whether the neural sub-type was differentially affected in these groups. Specifically, we were interested whether the odorant mixture activated a different ratio of excitatory and inhibitory neurons relative to anesthesia. We therefore double-labeled sections from groups AIR-SNT (A1), AIR-SNT(Anes) (A2) and AIR-AIR(Anes) (A4) in experiment 'A' with antibodies against c-Fos and GAD67, a marker of inhibitory neurons. Across all groups, we found relatively few c-Fos/GAD67 double-labeled cells in the anterior piriform cortex (overall AVG = 3.2% ± 0.3 of c-Fos$^+$ cells). Slightly greater co-labeling was found in the orbitofrontal cortex (overall AVG = 7.6% ± 0.5 of c-Fos$^+$ cells). A significant group effect (one-way ANOVA) was found in both the APC ($F_{(2,19)} = 5.47$; $p < 0.05$) and OFC ($F_{(2,18)} = 5.71$; $p < 0.05$). Tukey *post-hoc* test of the APC revealed a significantly greater percentage of double-labeled cells in the rats who received anesthesia alone (AIR-AIR(Anes), A4; Fig. 2-10) relative to those that received the odorant mixture alone (AIR-SNT, A1; $p < 0.05$). Interestingly, in the OFC, we found significantly fewer double-labeled cells in the anesthesia alone group (AIR-AIR(Anes), A4) versus the odorant mixture alone group (AIR-SNT, A1; $p < 0.05$). This suggests that the odorant mixture and ketamine/xylazine anesthesia may activate discrete populations of cells.

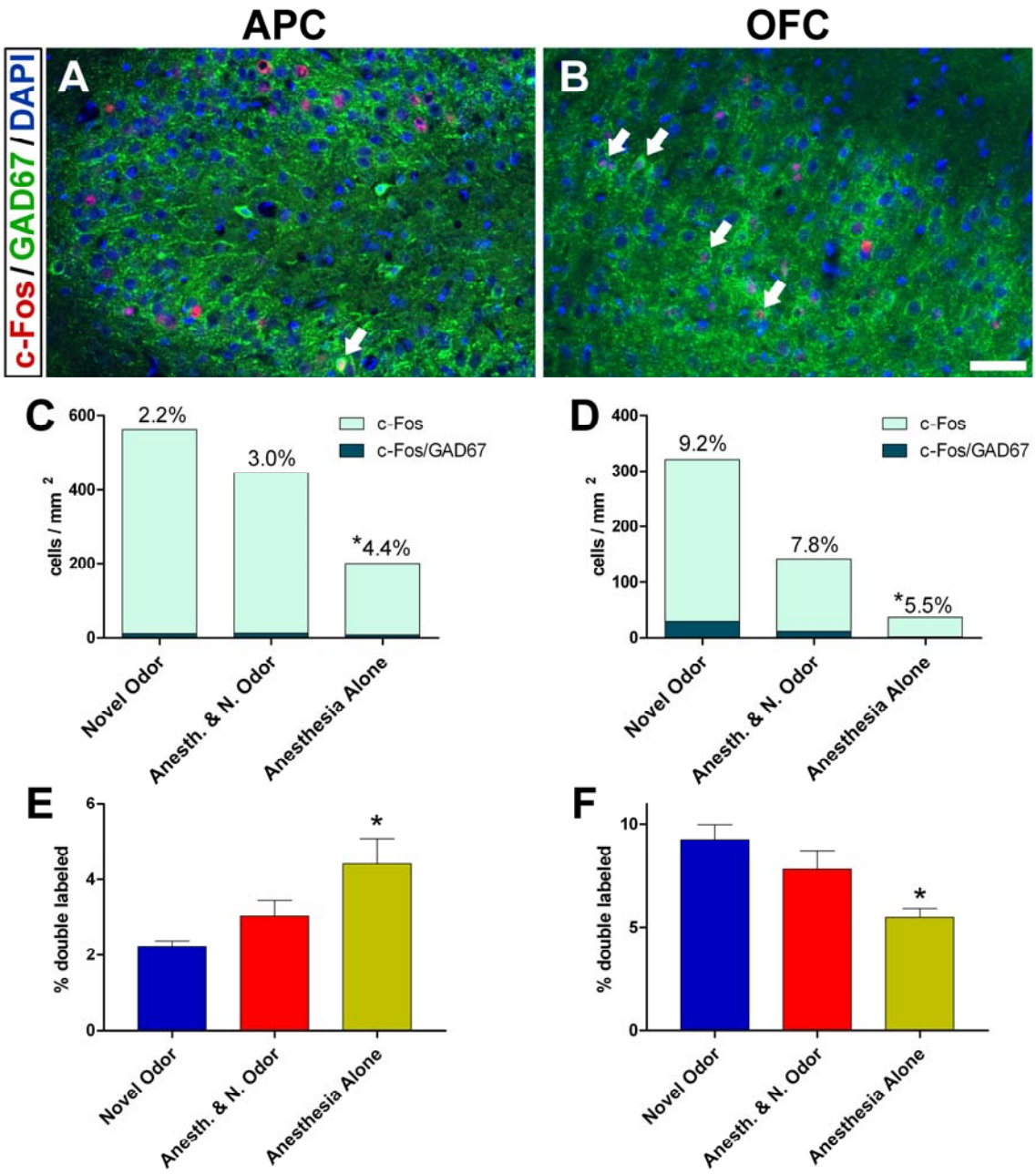

Figure 2-10. Overlay of c-Fos and GAD67 expression in the APC and OFC.

Fluorescent micrographs of c-Fos(red)/GAD67(green)/DAPI(blue) triple-labeling in the APC (A) and OFC (B). Double-labeled c-Fos/GAD67 cells are represented as a percentage of the total density of c-Fos$^+$ cells in the APC (C) and OFC (D). Group percentages from (C) and (D) are replotted in (E) and (F), respectively. Arrows indicate triple-labeled cells. Data from experiment 'A'. (*) $p < 0.05$ relative to Novel Odor group, one-way ANOVA with Tukey *post hoc* test. Scale bar = 50µm.

2.4 DISCUSSION

In our behavioral work we found that rats exposed to a novel odorant mixture while anesthetized on the training day produced a robust behavioral response to the same odorant mixture on the test day (Fig. 2-2). This response was indistinguishable from that of a novel odorant mixture. This suggests that olfactory memory could not be retrieved for events occurring under general anesthesia. These data are in line with a large body of literature showing profound multi-modal memory deficits resulting from anesthesia exposure (Weinberger et al., 1984; Ghoneim and Block, 1992; Pang et al., 1993; el-Zahaby et al., 1994; Alkire and Gorski, 2004; Bekinschtein et al., 2009). There are, however, some reports of a memory enhancing effect of very low-dose halothane and sevoflurane anesthesia (Alkire and Gorski, 2004; Alkire et al., 2005).

Explicit (or declarative) memory can be sub-divided into episodic memory, the autobiographical memory associated with an individual's personal experiences, and semantic memory, which is a general knowledge about the world unrelated to autobiographical experiences. Another sub-type of explicit memory is recognition memory (Squire et al., 2007), which is the type of memory that was behaviorally assessed in this study. Recognition memory is a type of long-term memory involved in determining whether a stimulus has been previously encountered. Exposure to a familiar person, object or experience is thought to be compared with a previously stored representation thus producing a matching signal (Norman and O'Reilly, 2003). Recognition memory is sub-divided into two components: recollection and familiarity. Recollection is thought to involve the memory of specific details of a previously experienced event, whereas familiarity is merely the knowledge that an event was previously experienced without any contextual details of the learning event (Brown and Aggleton, 2001; Squire et al., 2007). In this study we are interested in olfactory recognition memory, specifically that of odor

familiarity as we are unable to determine if further details of the odor experience are also present. In rodents, olfactory recognition memory is critically important as conspecific odors are thought to be the primary means of identification, similar to face recognition in humans (Schaefer et al., 2001). Furthermore, investigation into the underlying neurobiology of olfactory recognition memory has revealed the orbitofrontal cortex to be the critical region in mediating this process (Ramus and Eichenbaum, 2000).

Despite a lack of behavioral recognition memory, a histological imprint was nevertheless formed of the olfactory events that occurred under anesthesia. During general anesthesia, delivery of a novel odorant mixture activated c-Fos protein to a greater extent than anesthesia treatment alone (Fig. 2-5). This pattern suggests that the olfactory events were registered despite anesthesia. Not only was this information registered, but long-term changes appear to have occurred such that treatment with the same odorant mixture 24 hours later produced a 'familiar', or attenuated, c-Fos histological response comparable to that seen in awake animals exposed to a repeated odorant mixture (Fig. 2-8). Thus, the pattern of novel and familiar odorant exposure appeared similar in animals regardless of whether the odorants were experienced in the awake or anesthetized states. These histological data, in combination with our behavioral data, suggest an interesting distinction between what information can be recalled following anesthesia and what information is actually stored. In other words, a discrepancy may exist between what we know and what our brain knows regarding events that occurred during general anesthesia.

Expression of c-Fos in the olfactory and olfactory-association cortices was elevated after the presentation of a novel odorant mixture while in the awake state (Fig. 2-3). Our histological data showed repeated exposure to the same odorant mixture produced an attenuation of c-Fos expression in both the olfactory bulb and anterior piriform cortex. For Egr1, presentation of the

odorant mixture elicited elevated protein expression; however, both novel and familiar odorants produced equally elevated Egr1 expression relative to naïve animals, indicating that Egr1 reflects the presence or absence of the odor stimulus. Preferential expression of *c-fos* for novel (over familiar) stimuli mimics that found previously in the visual (Zhu et al., 1995; Zhu et al., 1996; Warburton et al., 2003), auditory (Kandiel et al., 1999; Wan et al., 2001), gustatory (Montag-Sallaz et al., 1999) and olfactory (Brennan et al., 1992; Hess et al., 1995b; Montag-Sallaz and Buonviso, 2002) systems, thus suggesting a role in recognition memory. However, expression of *c-fos* does not simply indicate novelty. For example, an odor-reward association task activates c-Fos in numerous odor processing regions but not when odorants and rewards are presented in an unpaired manner (Tronel and Sara, 2002).

Egr1 and *c-fos* are immediate early genes that are both activity dependent, but have varying expression profiles (Guzowski et al., 2001). It has been shown that *egr1* closely reflects the pattern of synaptic events whereas *c-fos* expression does not (Wisden et al., 1990; Abraham et al., 1991; Abraham et al., 1993; Gjerstad et al., 2005). While changes in synaptic strength (*i.e.* long-term potentiation; LTP) uniformly activate *egr1* (Abraham et al., 1991), *c-fos* expression may only be turned on during long-lasting, as opposed to short-lived, LTP (Kaczmarek, 1992). The expression profile of *egr1* has been shown to correlate with patterns of neural activity as demonstrated by 2-deoxyglucose (2-DG) labeling and optical imaging techniques (Inaki et al., 2002). As such, a number of studies have adopted the use of Egr1 as a marker of basic neural activity (Inaki et al., 2002; Mandairon et al., 2006b; Mandairon et al., 2008). In contrast, direct electrical stimulation, which elicits olfactory bulb mitral cell activity, fails to induce *c-fos* expression (Montag-Sallaz and Montag, 2006). Indeed, *c-fos* expression in the olfactory bulb has been shown not to parallel neural activity (Buonviso and Chaput, 1990; Sallaz and Jourdan,

1993), but to rely on top-down inputs as lesions to centrifugal afferents significantly reduce bulbar *c-fos* expression in response to odor. These lesions, however, do not attenuate the bulbar 2-DG response to the same odor (Sallaz and Jourdan, 1996). In fact, patterns of *c-fos* expression differ from 2-DG labeling throughout the brain (Duncan et al., 1998). Conversely, odorant stimulation of rodents with unilateral naris occlusion produces robust bulbar c-Fos expression both contralateral and ipsilateral to the occlusion (Jin et al., 1996). Unilateral lesions of the locus coeruleus produces a widespread reduction of cortical c-Fos expression ipsilateral to the lesion without any change in the EEG signal (Cirelli et al., 1996). As such, it is clear that *c-fos* expression encodes more than the activity state of the neuron. In contrast, *egr1* may more closely reflect the activity state of the neuron.

There are also a number of similarities between c-Fos and Egr1 as both genes are regulatory transcription factors thought to be involved in learning and memory (Guzowski et al., 2001; Guzowski, 2002). It is therefore possible that the continued high levels of Egr1 with familiar odors is a result of reconsolidation, that is, the process by which consolidation mechanisms are reactivated during re-exposure to a learning cue (Sara, 2000; Sara and Hars, 2006). However, this is unlikely as we did not see continued high expression of c-Fos with familiar odors as well, which we would expect to see if reconsolidation mechanisms recapitulated initial consolidation. Furthermore, the phenomenon of reconsolidation has been called into question as attempts to reproduce the original behavioral findings have failed (Dawson and McGaugh, 1969; Squire et al., 1976).

If *c-fos* were to reflect memory consolidation, one would expect changes in c-Fos expression to occur in tandem with behavioral changes. Previous work has shown that twenty minutes of odorant exposure for one week produces an attenuation in *c-fos* and *arc*

immunoreactivity to that odorant in the olfactory bulb (Montag-Sallaz and Buonviso, 2002), a finding that we have confirmed with c-Fos protein in various olfactory regions (Fig. 2-3, 2-8). However, we have found that it only takes a single 20 minute odorant exposure to produce a memory of the event 24 hours later (Fig. 2-4). Investigation of the pattern of odor-induced IEG expression following a single previous exposure confirms that levels of c-Fos protein are also attenuated at this point, a pattern that mimics the behavioral profile. As such, c-Fos is expressed in the appropriate temporal manner to reflect memory consolidation.

Indeed, expression of *c-fos* is reported to be a critical component of the neural plasticity leading to memory formation (Rose, 1991; Kaczmarek, 1993). Many studies have shown that *c-fos* can reflect memory consolidation. For example, infusion of the drug bicuculline into the accessory olfactory bulb, a process which has been shown to produce an artificial memory of male pheromones, also produces elevated *c-fos* expression (Brennan et al., 1992). Additionally, treatments that disrupt memory of visual images also abolish corresponding c-fos expression (Warburton et al., 2003). Noradrenergic fibers of the locus coeruleus descend into the olfactory bulb and provide a critical arousal component necessary for olfactory memories (Shea et al., 2008). Pharmacological inhibition of these noradrenergic afferents severely attenuates the bulbar *c-fos* expression in response to odors (Sallaz and Jourdan, 1996).

Other studies indicate that *c-fos* may have a causative role in memory formation. Impaired long-term memory formation is observed during *c-fos* knockdown (Lamprecht and Dudai, 1996; Mileusnic et al., 1996; Grimm et al., 1997; Morrow et al., 1999) and in *c-fos* knockout mice (Paylor et al., 1994; Guzowski, 2002; Fleischmann et al., 2003). Furthermore, memory impairments found in CNS-conditional *c-fos* knockouts are rescued when the *c-fos* gene is replaced by *fra-1* (Fos-related antigen 1), a transcription factor of the same family (Gass et al.,

2004). These studies demonstrate a critical and proactive role of *c-fos* in the information consolidation of memory events, and not merely a reflection of neural activity.

Knockdown of *c-fos* produced an impairment of a conditioned taste aversion memory. Interestingly, however, knockdown of *egr1* in the same region had no effect (Yasoshima et al., 2006). As discussed previously, *egr1* is expressed routinely during changes in LTP whereas *c-fos* requires long-lasting, robust LTP to be expressed (Abraham et al., 1991; Kaczmarek, 1992; Abraham et al., 1993). Furthermore, *c-fos* demonstrates low basal expression, whereas *egr1* is expressed at high basal levels (Terleph and Tremere, 2006). These studies indicate that *c-fos* expression requires a higher threshold of activity than that of *egr1*. The robust neural activity associated with memory formation is thus more likely to parallel changes in *c-fos* expression than *egr1*. A functional connection between *c-fos* and memory processes may explain why *c-fos* appears to be necessary for conditioned taste aversion memory, whereas *egr1* is not. Such a conclusion is consistent with the hypothesis that c-Fos reflects memory consolidation whereas Egr1 more closely reflects neural activity.

The aforementioned *c-fos* knockdown and knockout studies, in tandem with our current findings with c-Fos immunohistochemistry (Figures 2-5, 2-6 and 2-8), support the view that memory processes can occur in the anesthetized brain similar to how they occur in the awake brain. We speculate that this anesthesia-resistant memory consolidation occurred despite a low activity state as revealed by Egr1 immunolabeling. Further studies utilizing an electrophysiological or *in vivo* imaging approach would be required to confirm these observations. In a well known study, a false crisis was staged while patients were fully anesthetized for dental surgery and although there was no post-operative recall of the event, patients were able to describe the events that occurred verbatim while later under hypnosis

(Levinson, 1965). Similarly, amnesia of a memory event in mice caused by subanesthetic doses of propofol was shown to be reversed by pharmacological manipulation 30 minutes prior to a recall test (Pang et al., 1993). These studies suggest a sub-conscious, but potentially accessible, memory trace formed during anesthesia. Our data help to support and reconcile these curious findings.

We have found that subtle differences exist between the subtypes of cells activated by the odorant mixture or by ketamine/xylazine anesthesia alone. For both forms of stimuli, c-Fos appears to be activated primarily in neurons (Fig. 2-9). Of the c-Fos^{+} neurons, relatively few neurons were found to be inhibitory in nature (3.2%) in the APC, whereas a slightly greater percentage of c-Fos positive neurons were inhibitory in the OFC (7.6%; Fig. 2-10). Interestingly, ketamine/xylazine anesthesia and the odorant mixture activated c-Fos in slightly different populations of cells. In the APC, anesthesia alone activated c-Fos to a greater extent in inhibitory neurons than the odor stimuli, with the opposite found in the OFC. When a novel odorant mixture is given to a rat after week-long anesthesia, c-Fos does not become activated (Fig. 2-13) suggests that the brain may process odor and anesthesia stimuli in a similar manner. However, the difference in neural sub-type activated suggests a unique pattern of c-Fos activation for each stimulus. To further clarify the sub-type of neurons activated by the odorants or anesthesia, future studies may want to address potential differences in the morphology, distribution or connections with noradrenergic, dopaminergic or cholinergic signaling pathways.

We found an attenuated c-Fos response with repeated odorant administration in the APC and OFC in experiment 'B', but not in experiment 'A' (Fig. 2-4). This discrepancy likely results from the difference between seven days of repeated exposure (Exp. 'A') and only a single repeat exposure (Exp. 'B'). Both the anterior piriform and orbitofrontal cortices encode a moderate to

high amount of associative content (Roesch et al., 2007). It is therefore possible that compensatory mechanisms caused gradual changes in c-Fos expression, over the course of the week, to reflect this increasingly common element in the animal's environment.

Upon closer examination of our raw histological images (Fig. 2-5, 2-6) a greater intensity of antibody labeling was seen in animals receiving a novel odorant mixture during anesthesia than animals receiving a novel odorant mixture alone. This finding is in line with a previous *in vivo* electrophysiological study which has revealed that odors elicit a stronger signal-to-noise neural response, in mouse olfactory bulb mitral cells, while under ketamine/xylazine anesthesia than while awake (Rinberg et al., 2006). Future studies may want to address the significance of these findings.

In experiment 'A' we maintained the body temperatures of our rats between 36-38ºC to prevent hypothermia. A similar procedure was utilized in experiment 'B', though body temperature was inferred based on techniques used in experiment 'A'. The avoidance of hypothermia in our animals was important as it has previously been shown that hypothermia, produced by general anesthesia in the rodent (body temperatures between 26-30ºC), can lead to tau hyperphosphorylation, which was attributed to decreased protein phosphatase 2A (PP2A) activity (Planel et al., 2007). Restoring normal body temperature in these animals prevented the associated changes in tau phosphorylation and PP2A activity. In a related paper, it was shown that inhibited PP2A activity upregulated the transcription of a *luciferase* gene driven by the *c-fos* promoter (Johannessen et al., 2003). Taken together, these papers suggest a role of anesthesia-induced hypothermia in enhanced *c-fos* expression. Other physiological endpoints (*e.g.* blood gases, heart rate, respiration rate, etc.) were not directly quantified in these studies. The use of additional physiological monitoring devices was avoided to minimize the premature spread of

our novel olfactory cues (which may adhere to these devices) and to maintain the integrity of the odor-deprived environment. Though we did not directly quantify measures of respiration quality, these variables may also have a significant impact on IEG expression as hypoxia and elevated CO_2 (hypercapnia) are able to induce *c-fos* expression in various brain regions (Larnicol et al., 1994; Miura et al., 1994; Prabhakar et al., 1995; Haxhiu et al., 1996) including the piriform cortex (Dell'Anna et al., 1995). This is thought to occur by causing elevated Ca^{2+} entry through L-type voltage-gated calcium channels (Premkumar et al., 2000). However, based on related studies in our lab it was found that the color of the rat's skin tone could reasonably predict the oxygen saturation of the blood, with a pink tone producing greater than 90% SpO_2 (oxygen saturation; unpublished observation). We used this as an indirect measure of respiration quality and did not observe any incidents of insufficient respiration in experiments 'A' or 'B'. We recognize that administration of the anesthetic alone may alter the physiological state of the organism; however, invasive measures of physiological parameters could introduce other confounding variables to our analysis. Thus, to address the potential effects of the anesthetic on physiological measures and ultimately IEGs we ran control groups utilizing the anesthetic alone in both experiments 'A' and 'B' (groups AIR–AIR(Anes), A4, and AIR(Anes)–SNT, B4, respectively) to account for any effects of the anesthetic itself.

We did not find a significant effect between anesthetized animals receiving the odorants and those not receiving the odorants in the primary region of olfactory processing: the olfactory bulb (Fig. 2-7). It is well documented that ketamine anesthesia, and other NMDA antagonists, augment *c-fos* expression in various regions of the brain (Wilson et al., 1996; Duncan et al., 1998). In our study, ketamine/xylazine anesthesia alone produced a c-Fos response equal to or

greater than that of a novel odorant mixture in the olfactory bulb, thus obscuring any cumulative effects in that region.

Though there were no significant differences in anesthetic dose administered (one-way ANOVA followed by Tukey post hoc test), animals in the AIR-AIR(Anes) (A4) group received a slightly greater dose (170.8 mg/kg ketamine, 22.8 mg/kg xylazine) relative to AIR-SNT(Anes) (A2) animals (125.1 mg/kg ketamine, 16.7 mg/kg xylazine), though not significantly different (t-test, $p = 0.10$). It has been shown that greater doses of ketamine anesthesia correlate with greater c-Fos protein expression in cortical areas, including the orbital and piriform cortices (Duncan et al., 1998). As such, we expect our findings in Figures 2-5 and 2-6 to be even more robust with an identical anesthetic dosage.

Long-term memory formation can occur during anesthesia in some altered brain states. It has been shown that exogenous epinephrine can enable pavlovian conditioning under general anesthesia without altering the depth of anesthesia (Weinberger et al., 1984; el-Zahaby et al., 1994). More recently, memory of an odorant was shown to form in the anesthetized mouse when the odorant was presented with concurrent locus coeruleus stimulation (Shea et al., 2008). Sub-anesthetic doses typically produce amnesia (Pang et al., 1993; O'Gorman et al., 1998; Pitsikas et al., 2008); however, under certain circumstances, such as basolateral amygdala lesions (Tomaz et al., 1992; Alkire et al., 2001; Alkire and Nathan, 2005) or co-administration of cognitive-enhancing agents such as nefiracetam (O'Gorman et al., 1998), memories are still able to be formed. In line with these previous studies, our current findings support the notion that the brain may receive and retain information despite general anesthesia. Some degree of information consolidation may occur, even if there is no evidence of memory recall. This suggests that

anesthetic-induced amnesia may result from a deficit in retrieval or that initial consolidation mechanisms that do occur are incomplete.

3.0 CHARACTERISTICS OF BEHAVIORAL RECALL FOLLOWING ANESTHESIA

3.1 INTRODUCTION

Amnesia is one of the most profound effects of general anesthesia and even subanesthetic doses have been shown to produce profound memory loss (Block et al., 1988; Chortkoff et al., 1995; Imre et al., 2006). However, the assumption that memories are completely blocked may not be entirely correct. Growing evidence supports a view that initial memory consolidation may occur, and, under certain circumstances, can be recalled (Ghoneim and Block, 1992; Pang et al., 1993). Our initial tests on explicit olfactory memory revealed no evidence of memory for odorants presented during ketamine/xylazine general anesthesia. Although a clearly defined explicit memory was not apparent, we hypothesize that, based on histological changes in various brain regions under anesthesia, behavioral endpoints can still reveal some element of familiarity. These may be evident through subtle behavioral or autonomic processes and may occur contrary to what is explicitly displayed by the animals. We therefore studied whether an odorant, presented to animals under anesthesia, was reported as equally 'novel' as a completely novel odorant when the two were presented side-by-side. We wanted to test whether any subtle preference was present for the completely novel odorant. The test involved a discrimination task

where odorized wooden beads were placed side-by-side in the animal's home cage (Spinetta et al., 2008), and relative odor investigation time by the rodent was assessed.

Disruption of the consolidation processes following a memory event can prevent recall of the particular memory (Sara and Hars, 2006). These experimentally-induced amnesias can be produced through a number of methods including: anesthesia, electroconvulsive shock, carbon dioxide, hypothermia, disruption of neurotransmitter signaling and certain antibiotics and protein synthesis inhibitors (Flexner et al., 1963; Rigter and Van Riezen, 1979; Quartermain et al., 1988; Sara and Hars, 2006). It is possible to reverse some experimentally-induced amnesias by treatment with nootropic (cognitive-enhancing) or psychostimulant drugs prior to memory tests (Quartermain et al., 1988; Quartermain and Leo, 1988). These studies suggest that memory formation can occur despite the induction of amnesia. To date, the potential for memory consolidation and pharmacologically-induced retrieval has not been addressed in animals anesthetized at surgical doses. Thus, we also tested whether an anesthesia-paired memory could be re-elicited by pharmacological means.

In rodents, the racetam class of nootropic drugs has been found to be effective at enhancing memory retrieval, combating amnestic agents and reversing experimentally-induced amnesias (Sara and Lefevre, 1972; Sara and David-Remacle, 1974; Franklin et al., 1986) including anesthetic-induced retrograde amnesia (O'Gorman et al., 1998). The racetam class of drugs includes piracetam, nefiracetam, etiracetam, and pramiracetam and is thought to enhance cognitive function, in part, by working as allosteric modulators of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (Ahmed and Oswald, 2010). Piracetam is prescribed in humans for a variety of issues including cognitive disorders, dementia and dyslexia

(Winblad, 2005), and has been shown to boost memory function in healthy adults (Dimond and Brouwers, 1976).

Another cognitive-enhancing agent, amphetamine, has been shown to reverse amnesia induced by low dose anesthesia (Pang et al., 1993) as well as amnesia induced by electroconvulsive shock, protein synthesis inhibition or modulation of cholinergic, noradrenergic or serotonergic systems (Quartermain et al., 1988; Quartermain and Leo, 1988). Amphetamine is a psychostimulant that acts on the dopamine transporter (DAT) to increase synaptic dopamine and inhibit reuptake in the mesolimbic dopamine system (Leshner and Koob, 1999; Fleckenstein et al., 2007). Historically, amphetamine is known for its use by World War II pilots and commercial truck drivers to enhance cognitive function and alertness. Today, amphetamine derivatives are prescribed for the treatment of attention deficit hyperactivity disorder (ADHD) but are also commonly misused by college students as a study aid (Kollins, 2008). Several other pharmacological agents have also been shown to be effective at reversing amnesia including strychnine (Sara and Remacle, 1977), arecoline (Quartermain and Leo, 1988) and pituitary hormones (Rigter and Van Riezen, 1979). To assess whether memories of olfactory events possibly registered under general anesthesia could be retrieved, we treated animals with various cognitive-enhancing drugs and looked for any signs of intact memory formation.

In our current study we found that side-by-side comparison and pharmacological manipulation were unable to reveal any evidence of memory formation during ketamine/xylazine anesthesia, as determined by odor investigation time. Interesting effects in approach latency, however, suggested a potential implicit or visceral/autonomic memory.

3.2 MATERIALS AND METHODS

3.2.1 Experimental Animals

For all experiments, 7-week old male Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals were maintained in a temperature (20-22ºC) and humidity controlled environment with a constant 12:12 light-dark cycle (on 07:00 - 19:00 hr). Animals were individually housed to prevent incidental odors and allowed one week to acclimate to the colony and odor environment prior to treatment. The environmental odors in the housing facility were also strictly controlled to minimize any variation. Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

3.2.2 Experiment C: Odor Discrimination Task

Table 3-1. Group Layout: Experiment 'C'

Group	*Test*	*Odorant Combination*	*Pre-Exposure*	*Pharmacological Agent*	*n =*
C1	Odor Preference	Bn-Ci, Bn-Be, Bn-Wn, Ci-Be, Ci-Wn, Be-Wn	None	None	31
C2	Odor Recognition	Bn-Ci	Bn or Ci	None	14
C3	Odor Recognition	Bn-Ci	Anesthesia + Bn or Ci	None	24
C4	Odor Recognition	Bn-Ci	Anesthesia + Bn or Ci	Piracetam	12
C5	Odor Recognition	Bn-Ci	Anesthesia + Bn or Ci	Amphetamine	12
					Total = 93

3.2.2.1 Procedure

We adapted a previously described task (O'Dell et al.; Spinetta et al., 2008) for use with arbitrary odorants. Rats received various treatments according to Table 3-1. A total of 93 animals were used for this task. One week after arriving in the colony, rats began a 24-hour familiarization period. Each rat was handled for approximately 2 minutes, after which 4 round wooden beads were introduced into their home cage (day 1). Wooden beads were 1-inch in diameter with a 7/32" hole bore through the middle (Woodworks Ltd., Haltom City, TX) and individually coded with a black marker. The beads remained in the home cage overnight to allow the animals to become familiar with the 4 control beads (Fig. 3-1). The next morning (day 2), the beads were removed from the cage and the animal handled again for 2 minutes.

Testing for odor preference or odor familiarity occurred later in the same day. During the testing session the animal's home cage was placed in a hard-ducted hood that vented directly to the exterior of the building. Each animal was allowed to habituate to this environment for 10 minutes. Halfway through habituation, the lid of the cage was briefly removed and replaced to allow the ambient hood odors to fill the cage. After habituation, the 4 familiarized control beads were returned to the cage in a manner similar to the testing procedure (see below). The rats were allowed to freely interact with the control beads for five minutes, after which the beads were removed and the animal left alone for an additional 5 minutes. Odor preference or recognition testing began following the 5 minute delay period. The lid was removed and the animal was gently restricted to one end of the cage by hand. Two of the control beads were placed back in the cage as well as two novel, scented beads. The two novel beads were mildly scented with different odorants. The four beads were placed in a row (Odorant A, Control, Control, Odorant B) on the far side of the cage approximately 1-inch from the cage wall (Fig. 3-1). Each odorized

bead was placed on the far left or far right of two centrally positioned control beads to avoid ambiguity in the odor response. The rat was then released and allowed to freely interact with the 4 beads for 5 minutes. A video camera was positioned outside the clear plastic cage to record the animal's interaction with each bead. The left-right position of each odorant was reversed between trials.

Scented beads were produced by incubating wooden beads overnight in a 50ml Falcon tube (BD Biosciences, Bedford, MA) with 100 µl of odorant (diluted 1:10 in mineral oil) injected into a cotton reservoir in the bottom. Four different odorants were used (obtained from Sigma, St. Louis, MO): isoamyl acetate (banana, Bn), decanal (citrus, Ci), ethyl butyrate (berry, Be) and *R*-(-)-carvone (wintergreen, Wn; Table 3-1). A plastic insert prevented beads from directly contacting the scented oil. Any beads that came into direct contact with the oil were discarded. Pilot studies revealed that strongly scented beads were avoided or investigated from a distance. Our method produced beads with an odor that could barely be detected by the human nose. Rats actively and directly investigated beads scented in this manner.

Testing of odor preference was performed without any prior exposure to the specific odorants. For tests of odor familiarity, animals were previously exposed to a specific odorant on day 1 (Table 3-1). Pre-exposure of animals in the Awake condition occurred in a clean, empty cage within the hard-ducted hood. After acclimating to this environment for 5 minutes, two tubes of undiluted odorant (35µl in Eppendorf tube) were introduced into the cage in opposite corners and the animal allowed to interact with the odor for 20 minutes. The lid and odorant-filled tubes were then removed and the animal was allowed to air off in the hood for 15 minutes to whisk away lingering odors. Rats that were anesthetized then exposed to odorants received 10 seconds of potent exposure (tube 1" from snout) in the beginning, middle and end of the 20

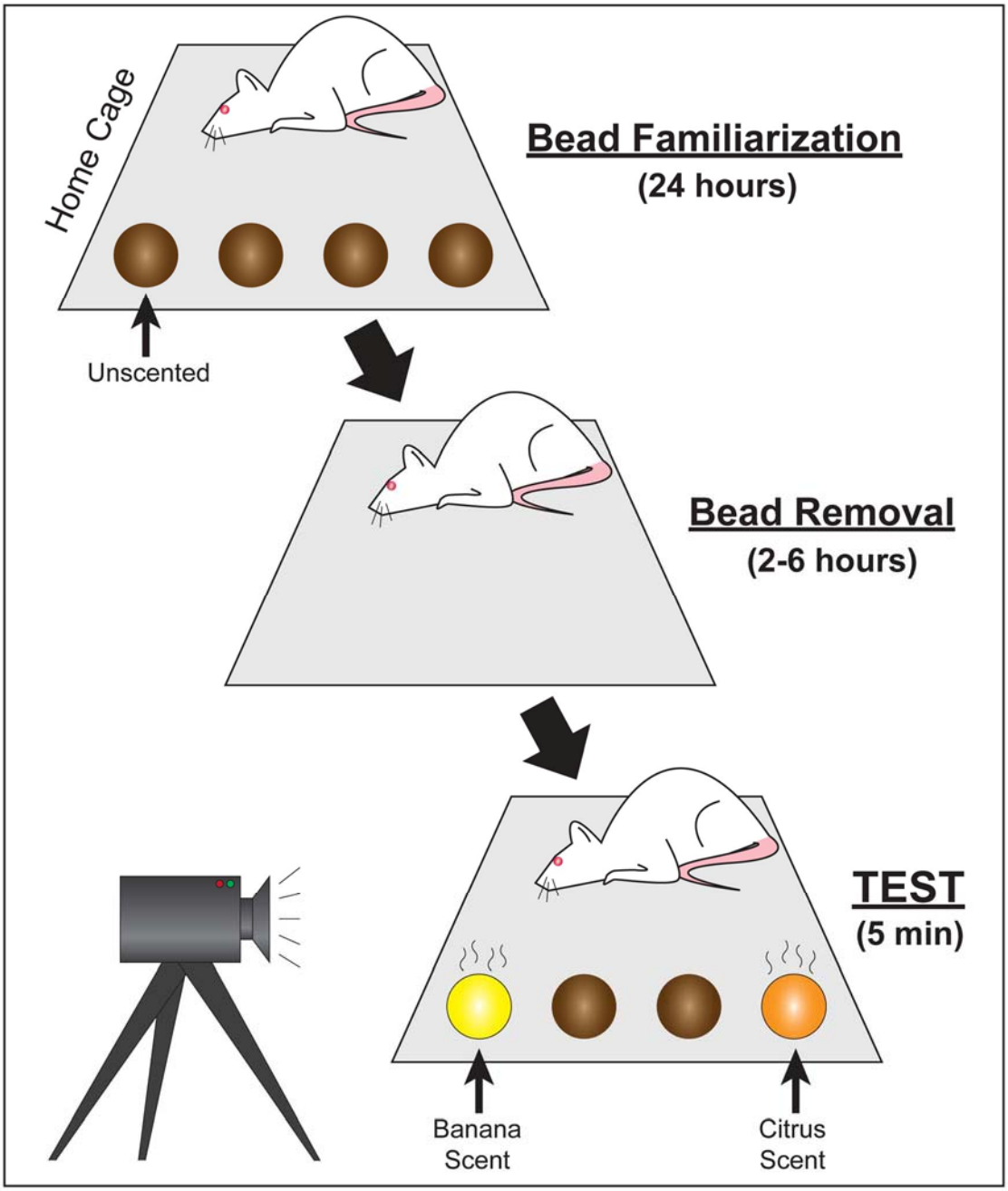

Figure 3-1. Simplified schematic of the odor discrimination task

Four unscented beads were placed in rat's home cage overnight to allow familiarization (Top). Beads were removed in the morning for several hours (Middle). Two beads scented with different odorants (typically banana and citrus; see section 3.3.1) and two unscented beads were reintroduced during testing (Bottom). Test behavior was video recorded and percentage of time smelling each bead was assessed. All procedure took place within the rat's home cage. Scented beads were placed on opposite ends of the two centrally located control beads. Beads are not drawn to scale.

minute period, and received moderate exposure (tube ≥ 5" from snout) for the remainder of the time. These animals were then placed on a heating pad within the hood and allowed to air off for 15 minutes before being returned to their home cage. Hypothermia was prevented by the combined use of lamps and heating pads positioned above and below the animal, respectively, throughout the period of anesthesia. Techniques established to maintain body temperature between 36-38ºC in experiment 'A' were utilized in experiment 'C'; however, direct measure of body temperature was not assessed.

3.2.2.2 Behavioral Analysis

Videos acquired during the behavioral task were assessed by a blinded observer. Duration of time spent smelling was assessed for the first 30 and 60 seconds after the animal approached each bead. Latency to approach was also assessed and capped at 30 seconds. Smelling behaviors (bead-directed sniffing, rearing) within 1-inch of the bead were positively scored. Time spent chewing, rolling or playing with each bead did not count towards smelling duration. Smelling duration over all beads was summed and investigation time of each bead was represented as a percentage of the total smelling time to normalize for innately shy or inquisitive animals.

3.2.2.3 Cognitive-Enhancing Agents

To aid in memory retrieval some groups were treated with cognitive enhancing agents as described in Table 3-1. Either piracetam (40 mg/kg; Sigma, St. Louis, MO) or d-amphetamine (1 mg/kg; Sigma) were administered 30 minutes prior to the odor familiarity test phase. These doses and times have been shown to be effective at enhancing memory retrieval (Franklin et al.,

1986; Quartermain and Leo, 1988; Pang et al., 1993). Piracetam and d-amphetamine were dissolved in sterile saline and administered through intraperitoneal injection.

3.2.3 Experiment D: Odor Attenuation Task

Table 3-2. Group Layout: Experiment 'D'

Group	*Habituation (days 1-3)*	*Training (day 4)*		*Pharmacological Agent*	*Testing (day 5)*	*n =*
D1	*AIR & Awake*	AIR	Awake	Amp – 2mg/kg - 30 min	*SNT & Awake*	4
D2	*AIR & Awake*	SNT	Anes	Amp – 2mg/kg - 30 min	*SNT & Awake*	4
D3	*AIR & Awake*	AIR	Awake	Str – 1mg/kg - 20 min	*SNT & Awake*	6
D4	*AIR & Awake*	SNT	Anes	Str – 1mg/kg - 20 min	*SNT & Awake*	(4) 1
D5	*AIR & Awake*	AIR	Awake	Nef – 10mg/kg – 1 hour	*SNT & Awake*	2
D6	*AIR & Awake*	AIR	Awake	Pir – 80mg/kg – 30 min	*SNT & Awake*	4
D7	*AIR & Awake*	SNT	Anes	Pir – 80mg/kg – 30 min	*SNT & Awake*	4
D8	*AIR & Awake*	AIR	Anes*	Str – 1mg/kg - 20 min	*SNT & Awake*	6
D9	*AIR & Awake*	SNT	Anes*	Str – 1mg/kg - 20 min	*SNT & Awake*	(6) 4
D10	*AIR & Awake*	AIR	Anes	K/X – 3mg/kg & 0.4 mg/kg – 20 min	*SNT & Awake*	3
D11	*AIR & Awake*	SNT	Anes	K/X – 3mg/kg & 0.4 mg/kg – 20 min	*SNT & Awake*	3
D12	*AIR & Awake*	AIR	Anes	Amp – 1mg/kg - 30 min	*SNT & Awake*	3
D13	*AIR & Awake*	SNT	Anes	Amp – 1mg/kg - 30 min	*SNT & Awake*	3
						Total = (52) 47

3.2.3.1 Procedure

The procedures in experiment 'D' were performed as described in experiment 'B' with the exception that all groups received injection of a cognitive-enhancing agent prior to the odor familiarity test. These experiments were conducted in the Rodent Behavioral Animal Core (RBAC) of the University of Pittsburgh. Experiments occurred in the dark with red-light illumination. A custom built 17x17" black Plexiglas floor was constructed to fit inside an open field activity monitor (model ENV-515, Med Associates, St. Albans, VT) of the same dimensions. A removable black Plexiglas cartridge, 2" in diameter, was positioned in the center of the floor, the profile of which acted as the 'odorant zone'. A 0.8-cm (diameter) hole in the center of the cartridge allowed indirect access to the odorant mixture from the testing field. The odorant mixture was contained in a 1.5-ml Eppendorf tube (Hauppauge, NY) that was positioned within the cartridge. The cartridge lay flush to the floor such that the animal could only detect the central hole. A clear Plexiglas lid was positioned over the chamber to contain odors and allow for video recording from above. Groups of rats received experimental treatments and pharmacological agents as described in Table 3-2. A total of 52 animals were used for the experiment. All animals not receiving intraperitoneal injection of anesthesia or a cognitive-enhancing drug were given 0.3ml saline injection 30 min prior to any behavioral work. Three animals in group D4 and two animals in group D9 were excluded from behavioral analysis due to seizure-like activity. The total original number of animals in each group, including those removed from analysis, is indicated in parentheses in Table 3-2.

Rats received a day of handling then three days of habituation in the chambers for twenty minutes each day. Halfway through the habituation, the cartridge was removed, the tube replaced with a new empty tube, and the cartridge was re-inserted. On training and testing days,

rats were first acclimated to the chamber with an empty tube in the cartridge for 15 minutes. After that, the cartridge was removed and the tube replaced with either an odorant-filled tube or a similar empty tube. During this experimental phase the rat was allowed to freely explore for a period of 5 minutes. The odorant mixture used during this period was diluted 1:1000 in mineral oil (Sigma-Aldrich, St. Louis, MO). 35 µl of the diluted odorant mixture was injected into a cotton reservoir at the bottom of the 1.5-ml Eppendorf tube. The odorant mixture used included equal parts of 6 different odorants (obtained from Sigma, St. Louis, MO) and included: isoamyl acetate, cineole, octanol, decanal, capronaldehyde, and *R*-(-)-carvone. Odorant-filled tubes were prepared ahead of time and stored in an air-tight container. Caution was used to prevent incidental premature odor exposure.

After the 5 minute trial animals were briefly returned to their home cage. They were then transferred to a clean, empty rat cage within a hard-ducted hood in a nearby room. Two tubes of the undiluted odorant mixture, or two empty tubes were then placed in the cage to ensure potent odor exposure similar to that administered in experiment 'A'. The rat was allowed to freely interact with the tubes for 20 minutes, after which they were aired off in a separate cage for 15 minutes before being returned to their home cages.

Several hours prior to receiving anesthesia on the training day, rats in the 'Anes' group first received an additional day of habituation to the behavioral chamber to ensure equal familiarity with the other groups. Later the animals were anesthetized and placed directly in an empty cage within the hard-ducted hood for exposure to undiluted odorants. These animals received a pulsatile odor exposure to mimic odor exploration while awake. Rats received 10 seconds of potent exposure (tube 1" from snout) in the beginning, middle and end of the 20 minute period, and received moderate exposure (tube ≥ 5" from snout) for the remainder of the

time. No tubes were used for anesthetized rats in the 'AIR' condition; however, they were otherwise treated identically. Anesthetized animals were then aired off for 15 minutes in the hood before being transferred back to their home cages. Lamps and heating pads were utilized to warm the animal as described in experiment 'A'; however, direct measure of body temperature was not assessed.

3.2.3.2 Behavioral Analysis

Video was acquired and analyzed using Limelight automated tracking software (v3.0; Actimetrics, Wilmette, IL) with black and white CCD cameras (Panasonic, Secaucus, NJ). Nose, body and tail locations were tracked using the software, and time of the nose within the pre-defined odorant zone was quantified automatically for the first 10 and 30 seconds from approach. The latency of rats to approach the odorant zone was assessed and capped at a maximum of 30 seconds. All animals in this experiment approached the odorant zone within the time allowed for the trial (5 minutes).

3.2.3.3 Cognitive-Enhancing Agents

Prior to testing on day 5, rats were administered pharmacological agents to aid in memory retrieval according to Table 3-2. The pharmacological agents used included (Sigma, St. Louis, MO): d-amphetamine (Amp, 2 mg/kg, 30 minutes prior and 1mg/kg, 7 hours prior), strychnine (Str, 1mg/kg, 30 minutes prior), nefiracetam (Nef, 10mg/kg, 1 hour prior) or piracetam (Pir, 80 mg/kg; 30 minutes prior). We also utilized low dose ketamine/xylazine anesthesia (K/X, 3mg/kg & 0.4mg/kg, 20 minutes prior). Comparable doses and times of drug administration have previously been shown to be effective at retrieval (Sara and David-Remacle, 1974; Sara and Remacle, 1977; Franklin et al., 1986; Quartermain et al., 1988; Quartermain and Leo, 1988; Pang

et al., 1993; O'Gorman et al., 1998; Yamada et al., 1999). Each drug was dissolved in sterile saline and administered through intraperitoneal injection.

3.2.4 Anesthesia

To ensure a uniform physiological state, rats were deeply anesthetized until they reached a surgical depth of anesthesia. 'Surgical depth' was defined as decreased respiration rate and depth, decreased muscle tone and absence of response to a noxious (repetitive toe pinch) stimulus. The anesthetic used was intraperitoneal injection of ketamine (Ketaset, Fort Dodge, Fort Dodge, IA; 100mg/ml) and xylazine (Anased, Lloyd Laboratories, Shenandoah, IA; 100mg/ml). An initial dose of anesthesia was used to immobilize the animals and subsequent booster injections were given until the appropriate plane of anesthesia was reached. During the training trial rats were given an average dose of 183.4 ± 4.9 mg/kg ketamine and 24.5 ± 0.7 mg/kg xylazine over an approximate 30 minute period in experiment 'C' and 173.7 ± 3.9 mg/kg ketamine and 23.6 ± 0.5 mg/kg xylazine in experiment 'D'. No significant differences were found between group averages for anesthetic dose administered in either experiment. Doses of ketamine and xylazine (± Standard Error, SE) by group for each trial in experiments C and D are represented in Table 3-3.

Table 3-3. Average dose of ketamine/xylazine anesthesia (mg/kg ketamine, xylazine ± S.E.).

Group	*Train*	Group	*Train*	Group	*Train*	Group	*Train*
C3	180.5 ± 7.5, 24.2 ± 1.0	**D2**	167.5 ± 3.7, 22.7 ± 0.6	**D8**	185.0 ± 15.8, 24.7 ± 2.0	**D11**	148.8 ± 3.1, 20.3 ± 0.5
C4	173.6 ± 8.0, 23.2 ± 1.1	**D4**	177.0 ± 16.6, 24.3 ± 2.3	**D9**	182.2 ± 5.6, 23.9 ± 0.8	**D12**	173.4 ± 21.8, 24.4 ± 3.0
C5	199.1 ± 8.8, 26.6 ± 1.2	**D7**	172.2 ± 8.4, 23.6 ± 1.4	**D10**	174.8 ± 6.8, 23.9 ± 1.2	**D13**	164.3 ± 5.0, 22.8 ± 0.6

3.2.5 Statistical Analysis

One-way ANOVA was used to determine a significant overall difference followed by Tukey's *post hoc* analysis for inter-group comparison. A student's t-test was used for experiments containing only two groups. Values are described as group means ± standard error. For all analyses, differences were considered significant when $p < 0.05$.

3.3 RESULTS

3.3.1 Adaptation of the odor discrimination task for arbitrary odorants

The odor discrimination task has previously been used to assess memory of conspecific rodents (O'Dell et al.; Spinetta et al., 2008). In these cases, beads that were incubated overnight in the animal's home cage were tested against beads that were incubated in the cages of various different rats. To exclude the complications of pheromones and the complex behaviors and brain activity associated with social cues (Dessi-Fulgheri and Lupo, 1982; Popik et al., 1991; Bannerman et al., 2001), and to remain consistent with our previous experiments, we attempted to apply arbitrary odorants to the odor discrimination task (Experiment 'C'; group C1). We first set out to find a pair of odorants for which rats showed a comparable affinity. The four odorants tested included: banana (Bn), citrus (Ci), wintergreen (Wn) and berry (Be). All six possible combinations of the four odorants were assessed (Fig. 3-2). The distribution of animals in each group evolved such that more animals were allocated to groups where the odor investigation time appeared to be more similar.

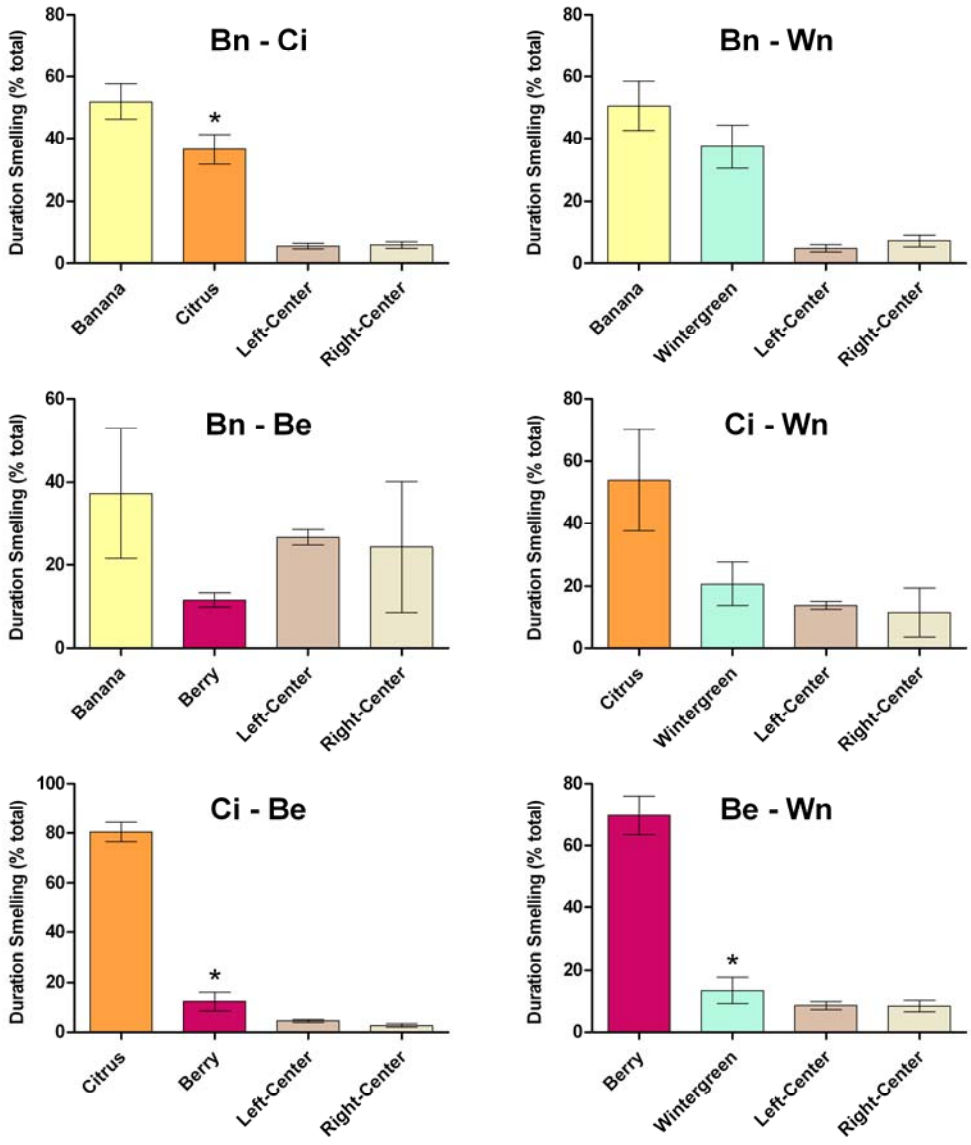

Figure 3-2. Specific odor preferences in the odor discrimination task

Relative odor investigation times between various pairs of arbitrary odorants. Pairs of odorant-scented beads were compared alongside non-scented control beads (Left-Center and Right-Center beads). Data from experiment 'C'. (*) $p < 0.05$ relative to leftmost bead (*i.e.* versus paired odorant). Smelling duration for each bead (within 1st minute of approach) represented as a percentage of the total smelling time for all four beads.

One-way ANOVA was used to assess the relative investigation time of all four beads (two scented, two unscented) for each odor combination. Tukey test was used for *post hoc* comparison of the two scented beads. Statistical data for each odor combination are represented in Table 3-4. The banana-citrus (Bn-Ci) and banana-wintergreen (Bn-Wn) combinations appeared to be the most comparable (see Fig. 3-2). We chose the banana-citrus combination for subsequent experiments as these appeared to be the two most favored odorants when paired against other odorants. However, banana did appear to be slightly favored and considerable variability existed in each rodent's odor preference. As such, the role of banana and citrus odorants were counterbalanced between each animal.

Table 3-4. Odor preference statistics

One-Way ANOVA:					
Bn-Ci	Bn-Wn	Bn-Be	Ci-Wn	Ci-Be	Be-Wn
$F_{(3,44)} = 37.27$	$F_{(3,40)} = 17.55$	$F_{(3,4)} = 0.88$	$F_{(3,4)} = 4.12$	$F_{(3,4)} = 180.4$	$F_{(3,12)} = 57.26$
$p < 0.0001$	$p < 0.0001$	$p = 0.52$	$p = 0.10$	$p = 0.0001$	$p < 0.0001$
Tukey *post hoc* test between the two scented beads:					
$p < 0.05$	$p = 0.33$	$p = 0.46$	$p = 0.21$	$p < 0.001$	$p < 0.001$
n = 12	n = 11	n = 2	n = 2	n = 2	n = 4

We also assessed the spatial preference for each bead over all groups in experiment 'C' (Fig. 3-3). Across all experiments, one-way ANOVA revealed a significant group effect ($F_{(3, 376)} = 139.3$; $p < 0.0001$). As expected, the left and rightmost positioned beads (odorized beads) were preferred over the center two control beads (non-odorized beads; $p < 0.001$ for all pair-wise comparisons). However, tukey post-hoc test revealed no significant differences between left and rightmost odorized beads ($p = 0.86$, tukey posttest), or left-center and right-center non-odorized, control beads ($p = 0.997$). This suggests that the spatial position of the beads during the task did not influence the rat's smelling behavior.

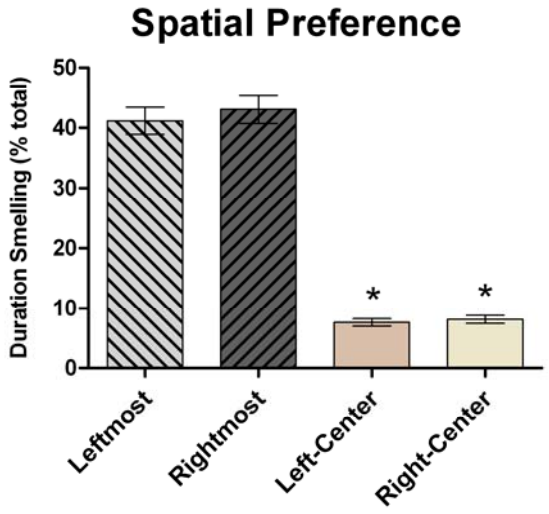

Figure 3-3. No spatial preference in the odor discrimination task

Relative odor investigation time as a function of bead location within cage. Scented (Leftmost and Rightmost) beads were compared alongside non-scented control beads (Left-Center and Right-Center beads). Spatial preferences were compiled over all groups in experiment 'C' (n = 95). (*) $p < 0.001$ relative to leftmost and rightmost beads. Smelling duration for each bead (within 1st minute of approach) represented as a percentage of the total smelling time for all four beads.

3.3.2 Absence of anesthesia-paired memory in the odor discrimination task

In order to validate the odor discrimination task using arbitrary odorants, we exposed rats to either a banana or citrus odorant on day 1. On day 2, rats were presented with both banana and citrus scented beads as well as two unscented, control beads and odor familiarity was assessed based on relative odor investigation time (Experiment 'C'; group C2). Rodents will spend more time investigating a novel odor compared to a familiar odor (Mandairon et al., 2006a). One-way ANOVA revealed a significant effect of smelling duration for all four beads ($F_{(3,52)} = 122.7$; $p < 0.0001$; Fig. 3-4A). Tukey post-hoc test revealed that rats spent significantly more time investigating the bead scented with a novel odorant than the bead scented with the odorant that

was presented on the previous day (familiar odor; $p < 0.001$). These results indicate that the odor discrimination task is a robust measure of odor familiarity even with arbitrary odorants, and that rodents display unequivocal familiarity with an odorant that was presented 24 hours previously.

We then asked whether an odorant, delivered during ketamine/xylazine anesthesia, would be reported as equally novel as a completely novel odorant (group C3). Rats were anesthetized and administered twenty minutes of either banana or citrus odorants, then tested with both odorants the following day. One-way ANOVA indicated a significant effect across all beads (Fig. 3-4B; $F_{(3,92)} = 67.24$; $p < 0.0001$). However, no significant differences were found between the novel odorant and the anesthesia-paired odorant ($p = 0.31$, tukey posttest). These data suggest that no memory of the anesthetic-paired odorant is evident in the odor discrimination task despite side-by-side comparison with a completely novel odorant.

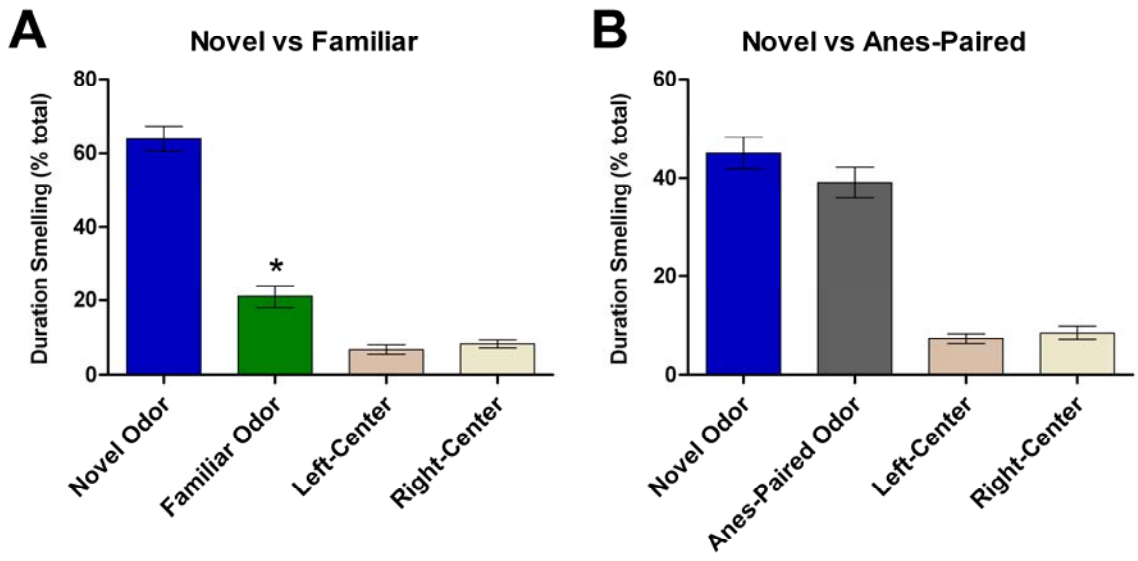

Figure 3-4. Absence of familiarity for anesthesia-paired odorants in the odor discrimination task

Odor investigation time between novel and familiar (A) or novel and anesthesia-paired (B) odorants relative to control, unscented beads (Left-Center and Right-Center). Data from experiment 'C'. (*) $p < 0.001$ relative to Novel Odor bead. Smelling duration for each bead (within 1st minute of approach) represented as a percentage of the total smelling time for all four beads.

3.3.3 Screening of cognitive-enhancing pharmacological agents

Many pharmacological agents have been labeled as cognitive-enhancing or nootropic drugs. A number of these have been shown to ameliorate or reverse amnesia (Sara and Remacle, 1977; Franklin et al., 1986; Pang et al., 1993). To date, the use of cognitive-enhancing agents to restore memories potentially acquired during surgical doses of general anesthesia has not fully been explored. To assess the possibility of olfactory memory during anesthesia, rats were fully anesthetized with ketamine/xylazine anesthesia and given twenty minutes of exposure to the odorant mixture. The following day rats were given an injection of a cognitive-enhancing agent then tested for familiarity with the odorants (Experiment 'D'). These rats were initially compared with rats also given the same drug prior to the test trial, but trained with 'Air' only.

Table 3-5. Statistical analysis of comparable drug treatments (student's t-test)

D1 vs D2 Amph	D3 vs D4 Strych	D5 Nefir	D6 vs D7 Piracetam	D8 vs D9 Strych	D10 vs D11 Ket/Xyl	D12 vs D13 Amph
$p = 0.44$	n/a	n/a	$p = 0.51$	$p = 0.48$	$p = 0.70$	$p = 0.63$

We first tested amphetamine (2mg/kg) delivered 30 minutes prior to the familiarity test (Fig. 3-5A). No significant differences (t-test, $p = 0.44$; Table 3-5) were found in the smelling durations of animals that were trained with air (group D1) and those trained with scent/anesthesia (D2); however, both groups paid little attention to the odorant stimulus and appeared hyperactive following amphetamine treatment. Compared to novel scent (non-drug) controls from experiment 'B' (B2; open grey bar, Fig. 3-5A), odor investigation times for both groups treated with amphetamine (D1 and D2) were significantly attenuated ($p < 0.05$; student's t-test). We

concluded that doses of amphetamine (2mg/kg) that were best associated with memory retrieval (Quartermain and Leo, 1988) also impaired performance on the odor attenuation task. We next tested strychnine (1mg/kg), administered 20 minutes prior to the familiarity test. All six control strychnine animals (D3) performed adequately and were not significantly different than non-drug controls (B2 and B5). However, rats that received scent/anesthesia (D4) during training displayed marked seizures in 3 of 4 animals, approximately 10-15 minutes following strychnine injection. This indicated a potential interaction between strychnine, a glycine antagonist, and residual ketamine/xylazine anesthesia from the previous day. As a result, we discontinued this group. Two animals were given nefiracetam (10mg/kg) one hour prior to the test (D5); however, this group was discontinued due to solubility issues of nefiracetam in saline. We next tested piracetam (80mg/kg) given 30 minutes prior to the familiarity test. No significant differences were found between groups receiving piracetam treatment (D6 and D7; $p = 0.51$, t-test) even though both groups displayed a robust, 'novel' behavioral phenotype, similar to groups B2 and B5.

Due to potential interactions of our cognitive enhancing drugs with residual anesthesia, as demonstrated by strychnine-induced seizure activity, we used animals trained with anesthesia alone as our control group for the remaining drug-treatments (Fig. 3-5B) to ensure that all groups were exposed to both the anesthetic and the cognitive-enhancing drug. We again tested strychnine (1mg/kg) delivered 20 minutes before the odor test. This time, rats were trained 2 days prior to the test to reduce the residual anesthesia and possibility of seizures. Only two of the 12 rats displayed any seizure-like activity (compared to 75% previously). Of the remaining animals, no significant differences were found between rats trained with scent and anesthetic (group D9) or the anesthetic alone (D8) in odor investigation time (t-test, $p = 0.48$), suggesting

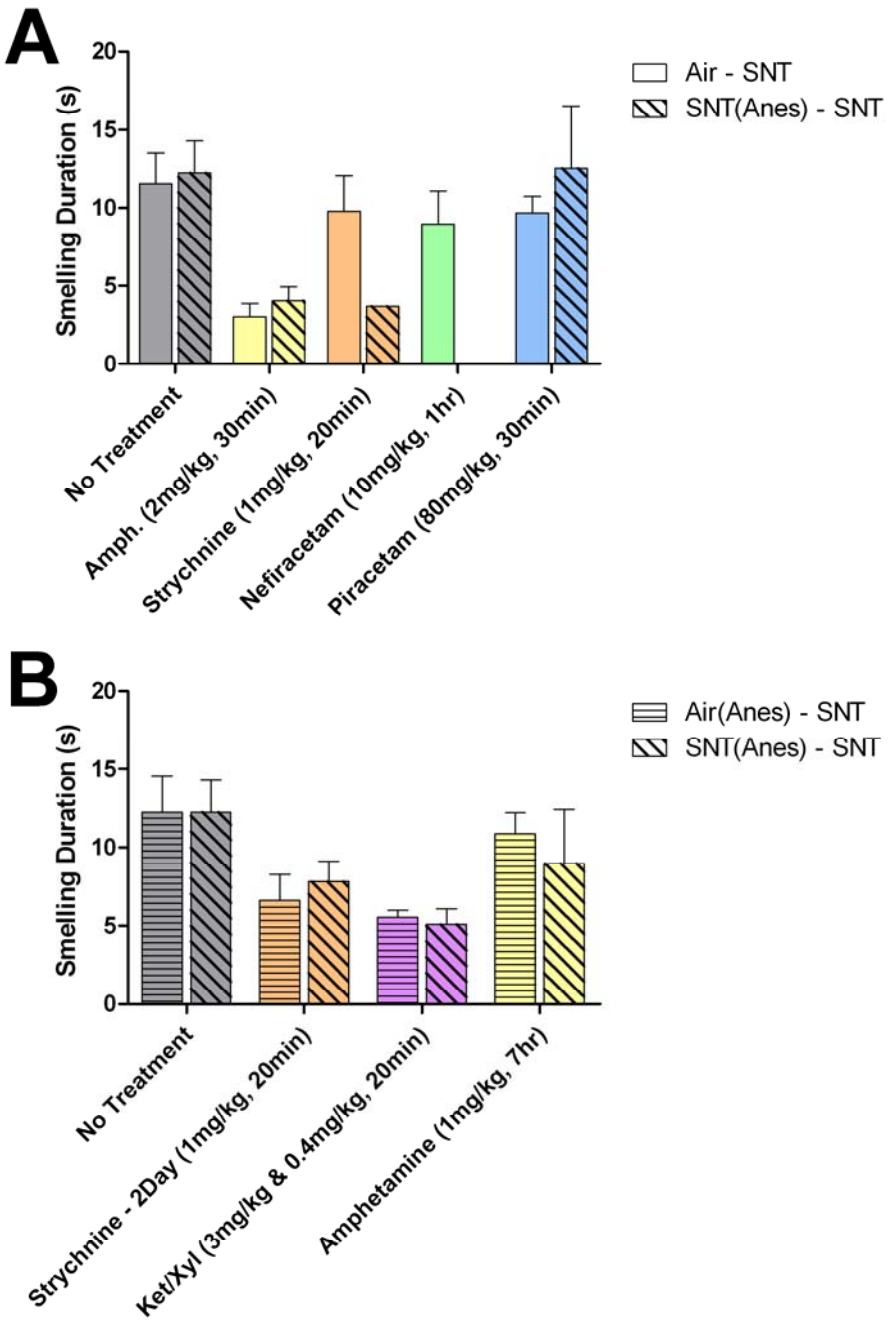

Figure 3-5. Effects of cognitive-enhancing drugs on memory retrieval

Duration of odor investigation following treatment with various memory enhancing drugs. Animals trained with scent while anesthetized were compared against animals trained with air alone (A) or air and anesthesia (B). Dosage and timing (prior to testing) of drug received are indicated in parentheses. Data from experiment 'D'. Smelling duration was measured for the first 30 seconds after approaching the odorant zone. Amph = amphetamine, Ket/Xyl = ketamine/xylazine anesthesia.

that strychnine was unable to reverse the anesthetic-induced amnesia. To elucidate potential state-dependent effects, we treated animals with low-dose ketamine/xylazine anesthesia prior to the test to see if this might aid in memory retrieval; however, no significant differences were found between animals trained with scent/anesthesia (D11) or anesthesia alone (D10; $p = 0.70$, t-test). Lastly, we again tested amphetamine treatment; however, this time at a lower dose (1mg/kg) delivered 7 hours prior to the task. No significant differences were found between groups (D12 and D13; $p = 0.63$, t-test) despite a robust behavioral response (absence of hyperactivity and distractibility). These results suggested that none of the cognitive-enhancing agents tested were able to reverse anesthesia-induced amnesia in the odor attenuation task, as assessed by the duration of odor investigation.

3.3.4 No effect of pharmacological manipulation on the odor discrimination task

Though treatment of rats with cognitive-enhancing pharmacological agents was unable to reverse anesthetic-induced amnesia in the odor attenuation task (Fig. 3-5), it is possible that these agents may produce a more subtle effect only observable in the odor discrimination task (experiment 'C'). We chose to utilize piracetam in the odor discrimination task (group C4) as this is a well documented memory-enhancing drug (Sara and David-Remacle, 1974; Franklin et al., 1986) that did not produce any confounding behavioral effects in the odor attenuation task. We also found a possible effect in approach latency with this drug (see Section 3.3.5). We compared the odor investigation time between an anesthetic-paired odor bead, a novel odor bead and two control (left-center and right-center) beads in rats pretreated with piracetam (40 mg/kg) 30 minutes before the test. One-way ANOVA revealed a significant effect of investigation time across beads ($F_{(3,44)} = 20.98$; $p < 0.0001$; Fig. 3-6A); however, pair-wise comparison of novel and

anesthetic-paired odorants revealed no significant differences in piracetam-treated animals (p = 0.995; tukey posttest).

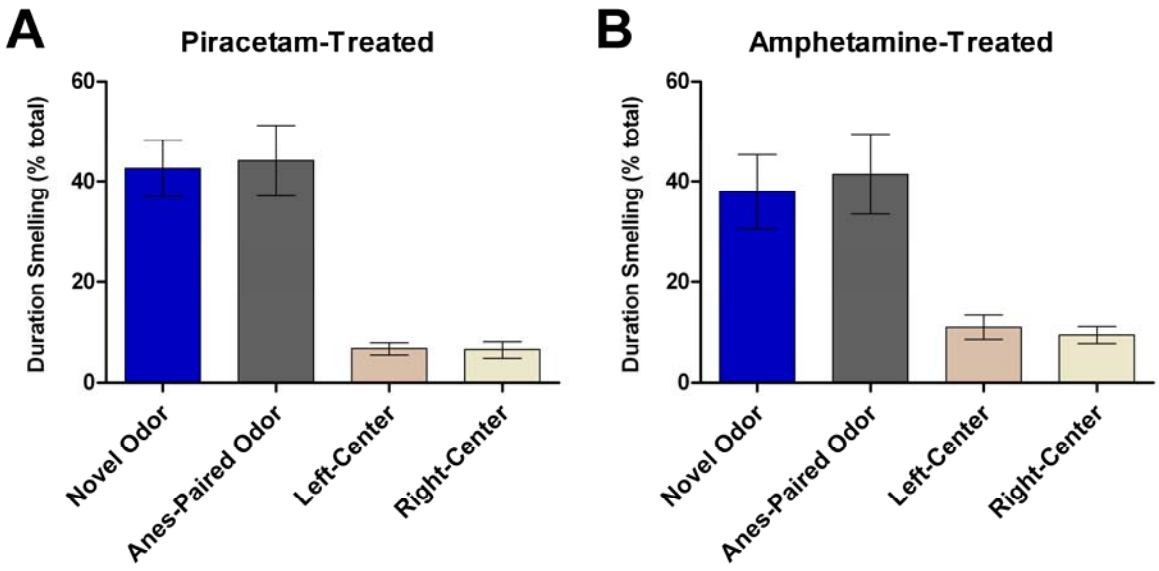

Figure 3-6. Effects of pharmacological manipulation on the odor discrimination task

Smelling duration between novel and anesthesia-paired odorants for piracetam (A) and amphetamine (B) treated animals. Piracetam (40mg/kg) and amphetamine (1mg/kg) were delivered 30 minutes prior to testing. Data from experiment 'C'. Smelling duration for each bead (within 1st minute of approach) represented as a percentage of the total smelling time for all four beads.

We also investigated the memory-enhancing capabilities of amphetamine in the odor discrimination task (group C5). Amphetamine was used as it is also a well documented memory enhancer (Quartermain et al., 1988; Quartermain and Leo, 1988) that has specifically been shown to reverse anesthetic-induced amnesia (Pang et al., 1993). We similarly compared the odor investigation time of each bead for rats administered amphetamine (1mg/kg) 30 minutes prior to the memory test. We found a significant effect of investigation time over all beads ($F_{(3,44)}$ = 9.22; $p < 0.0001$; one-way ANOVA; Fig. 3-6B); however, we did not find a significant effect between novel and anesthetic-paired odorants (p = 0.97; tukey posttest). These data

suggest that neither piracetam nor amphetamine treatment were able to restore explicit memory of anesthetized events as determined by odor investigation time.

3.3.5 Possible memory of anesthesia-paired odorants through indirect measures

Latency to approach the odor stimulus was assessed as a second and indirect measure of odor familiarity. Analysis of approach latency in tandem with pharmacological manipulation produced some interesting results. During our screening of pharmacological agents (experiment 'D'), we found a significant difference in the approach latency of piracetam-treated rats trained with scent/anesthesia (SNT(Anes)-SNT, group D7; Avg. = 23.4 s ± 4.1) and piracetam-treated rats trained with air alone (AIR-SNT, group D6, Avg. = 8.4 s ± 4.4; student's t-test; $p < 0.05$; Fig. 3-7A). These data suggest that piracetam treatment attenuated the urgency of odor investigation in rats previously exposed to the odorant while anesthetized; however, net odor investigation time remained unaffected. No other significant latency effects were found for groups in experiment 'D'.

In the odor discrimination task (experiment 'C'), we found a significant difference between groups (C2-C5) in latency to approach the first bead (Fig. 3-7B; $F_{(3,57)} = 2.79$; $p < 0.05$; one-way ANOVA). *Post-hoc* comparison revealed that rats trained with an odorant under anesthesia (Novel vs Anes-Paired, group C3) appeared to have longer approach latencies (Avg. = 8.25 s ± 1.38) than rats trained with the odorants while awake (Novel vs Familiar, group C2; 4.89 s ± 0.52); however, this effect was not significant ($p = 0.15$; tukey posttest). Interestingly, rats trained with an odorant under anesthesia, but pretreated with the cognitive-enhancing agents piracetam (Novel vs Anes-Paired (PIR), group C4) or amphetamine (Novel vs Anes-Paired (AMPH), group C5) prior to the test displayed similar approach latencies (4.55 s ± 0.86 and 4.80

s ± 0.78, respectively) to rats trained without anesthesia (C2 vs C4: $p = 0.998$; C2 vs C5: $p = 1.00$; tukey posttest). However the reduction in approach latency for rats pretreated with memory enhancing drugs did not reach significance (C3 vs C4: $p = 0.12$; C3 vs C5: $p = 0.16$; tukey posttest). These findings suggest a potential indirect memory of the anesthesia-paired odorants produced by the administration of either piracetam or amphetamine.

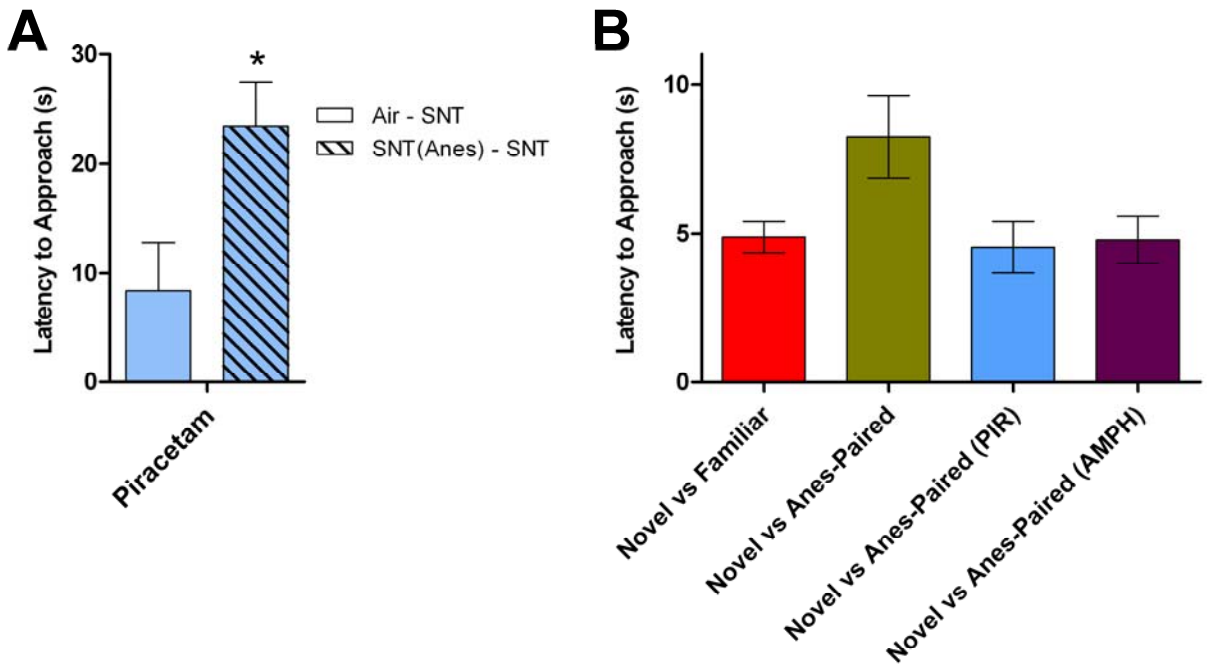

Figure 3-7. Latency effects in experiments 'C' and 'D'

Approach latency between rats treated with piracetam and tested with novel or anesthesia-paired odorants in the odor attenuation task (A). Differences in approach latency for groups in the odor discrimination task (B). Data from experiments 'C' and 'D'. (*) $p < 0.05$ relative to Air-SNT group. PIR = piracetam; AMPH = amphetamine.

It should be noted that approach latency in the odor attenuation task was highly variable and did not appear to be a robust measure of odor familiarity. No significant effects were found for approach latency in experiments 'A' or 'B'. Although, the number of animals in experiments

'A' and 'B' was lower (odor attenuation task; n = 6-7) than that in experiment 'C' (odor discrimination task; n = 12-24), which may contribute to the greater variability.

3.4 DISCUSSION

In the odor discrimination task, rats were forced to choose between a completely novel and an anesthetic-paired odorant. This allowed us to test whether anesthetic-paired odorants, reported as 'novel' in the odor attenuation task, were reported as *equally* interesting relative to a completely novel odorant. The side-by-side nature of both odorants in the odor discrimination task allowed us to tease apart more subtle distinctions than that found in the odor attenuation task. We validated that memory formation could occur with arbitrary odorants in the odor discrimination task; however, we did not find any evidence of explicit memory for events occurring under anesthesia.

Subanesthetic doses of general anesthesia can produce experimentally-induced amnesia, a type of memory loss that can be reversed by treatment with cognitive enhancing agents (Pang et al., 1993; O'Gorman et al., 1998). We therefore screened a number of cognitive-enhancing agents, using the odor attenuation task, to assess their potential memory enhancing effects for events occurring under surgical doses of general anesthesia. We found no differences between the odor investigation times of novel and anesthetic-paired odorants for all pharmacological agents tested. However, animals pretreated with piracetam appeared less eager to approach an odorant mixture if it was previously delivered to the animal under anesthesia. Once the odorant mixture was approached, however, rats spent an equal amount of time investigating the anesthesia-paired odorant mixture as they would a novel odorant mixture.

Based on our initial screening of cognitive-enhancing agents, we utilized piracetam and amphetamine (though no effects were found for this drug) in conjunction with the odor discrimination task in hopes that the memory-enhancing drugs would facilitate retrieval when novel and anesthetic-paired odorants were presented side-by-side. Treatment with either agent had no effect on the relative investigation time of novel and anesthetic-paired odorants. Both odorants were reported as equally 'novel' for each treatment, thus indicating no memory of the odorant administered during anesthesia. However, both piracetam and amphetamine appeared to normalize approach latency to that found in non-anesthetized animals, a finding that may reflect some level of odor recognition.

One interpretation of the effects on approach latency in the odor discrimination task (Fig. 3-7) is that the presentation of both anesthesia-paired and completely novel odorants produces an indecisive behavioral phenotype in the rat. The animal may have difficulty choosing the appropriate odorant to approach as both odorants seem to be 'novel'. Previous exposure to the odorant while in the awake state appears to clarify this ambiguity and the animal readily approaches the odorants. Similarly, pretreatment with cognitive enhancers, in animals trained with scent/anesthesia, also reconciles the ambiguity. In this case, now the animal 'knows' that the odorants are either novel or familiar. Regardless, these data reveal a potential memory trace not be evident in our tests of explicit olfactory memory (odor investigation time). This supports the hypothesis that some level of consolidation occurred in these animals. It is unknown, however, whether explicit memory was absent due to incomplete information consolidation or a deficit in memory retrieval.

In contrast to the explicit odor recognition (familiarity) memory observed in animals previously exposed to the odorants while awake, the interesting latency effects in the odor

attenuation and odor discrimination tasks suggest a possible implicit or visceral/autonomic familiarity response to an anesthetic-paired odorant. An "implicit" memory may best describe our observed effects as task latency has previously been used as a measure of implicit memory in mice (Trueman et al., 2005). Furthermore, latency effects appeared to occur in our study in the absence of conscious recall, which further suggests a role in implicit memory. It is nonetheless possible that effects seen with latency actually utilize neuronal processes and pathways distinct from "implicit" memory. Human studies have revealed that novel and familiar odors can produce unique physiological responses. Specifically, repeated odorant administration produces autonomic changes in facial electromyography, heart rate and abdominal respiratory amplitude (Delplanque et al., 2009). To date, the use of these autonomic functions as a measure of anesthesia-resistant memory has not been assessed, but provides direction for future study. Previous clinical studies have indicated that some implicit memory formation may occur during anesthesia (Block et al., 1988; Iselin-Chaves et al., 2005). In one study, patients were fully anesthetized with propofol, after which the dose was briefly lowered and individuals were allowed to return to a verbally responsive state. The patients were then presented with a picture, a sound and an odorant and re-anesthetized. Though explicit memory was later absent in all patients, approximately a third of the patients reported "… vague sensations, one of a colour, one of a smell and one of a sound (Barr et al., 2001)." These reports seem to echo that found in the blindsight literature, where patients describe an astoundingly accurate 'feeling' of an object's appearance or movement, despite visual cortical defects that produce complete perceptual blindness (Weiskrantz et al., 1974; Cowey, 2010). Interestingly, these blindsight patients also have intact neuroendocrine and reflexive responses when exposed to visual stimuli (Stoerig and

Cowey, 1997), a finding that further suggests a potential anesthesia-resistant memory in the form of intact visceral/autonomic functions.

The use of strychnine has been shown to be effective at attenuating amnesia induced by electroconvulsive shock (Sara and Remacle, 1977). We did not find any effects of strychnine on enhancing the retrieval of an anesthetic-paired odor memory (Fig. 3-5). However, in order to avoid interactions between strychnine and ketamine/xylazine anesthesia, rats were trained two days prior to testing. It is possible that simple olfactory recognition memory is no longer intact at 48 hours post-training and this produced a false negative result with strychnine treatment. This conclusion is unlikely given the lack of effects we found for the other cognitive-enhancing drugs. Also, early pilot studies revealed odorant recognition present several days after the initial exposure (experimenter observation).

An early study performed by an oral surgeon demonstrated that patients were unable to remember events that occurred during anesthesia; however, these memories were able to be retrieved later during hypnosis (Levinson, 1965). This suggests a potential state-dependent retrieval effect. We chose to test this by using a subanesthetic dose of ketamine/xylazine (3mg/kg and 0.4mg/kg, respectively), in attempt to induce state-dependent effects. This dose was shown to affect memory processes without altering task performance (Pitsikas et al., 2008). At this dose, our animals performed the task adequately; however, no memory of the anesthetic-paired odorant mixture was elicited. It is therefore possible that state-dependent retrieval does not occur with ketamine/xylazine anesthesia. Alternatively, the low dose anesthesia used may not have been sufficient to recreate the state under which the memory would have been acquired. Indeed, a dichotomy exists between subanesthetic and fully anesthetic doses of ketamine in brain activity and autonomic response (Duncan et al., 1998; Langsjo et al., 2005), suggesting that the

retrieval state may not have reflected the acquisition state. As such, it is possible that a state-dependent retrieval effect may still be observed at higher anesthetic doses than that which was utilized in this experiment.

Using the pharmacological agents piracetam, amphetamine and strychnine, we were unable to find evidence of explicit memory retrieval following anesthesia. Other agents have shown promise in reversing amnesia and alleviating memory deficits including nefiracetam (O'Gorman et al., 1998; Yamada et al., 1999), pituitary hormones (Rigter and Van Riezen, 1979), GSK189254 (Foley et al., 2009), and arecoline (Quartermain and Leo, 1988). Epinephrine (Weinberger et al., 1984) and norepinephrine (Shea et al., 2008) have been shown to facilitate memory formation under general anesthesia and may also enhance subsequent retrieval. Future studies could address the potential for these agents to restore memories of anesthetic-paired events. We did find some level of initial information consolidation with piracetam and amphetamine as evident by subtle effects in approach latency. These data provide interesting evidence that information received during anesthesia can be reflected in subsequent behavior.

4.0 SPECIFICITY OF HISTOLOGICAL CONSOLIDATION

4.1 INTRODUCTION

In chapter 2 we found that administration of a novel odorant mixture during ketamine/xylazine anesthesia caused an activation of c-Fos protein similar to that which occurred in awake animals. This may indicate that certain mechanisms of memory consolidation are activated during anesthetic-induced unconsciousness. It is unknown whether this effect is specific to ketamine/xylazine anesthesia or is a universal trait amongst anesthetics. To address the ubiquity of ketamine/xylazine effects, potential histological consolidation was assessed with pentobarbital and propofol anesthesia. Pentobarbital and propofol are ideal anesthetics as both these drugs differ in their proposed mechanisms of action from ketamine, which primarily acts through inhibition of the *N*-Methyl-$_D$-aspartic acid (NMDA) receptor (Harrison and Simmonds, 1985; Martin and Lodge, 1985). Pentobarbital is a barbiturate anesthetic commonly used in veterinary medicine, that has strong effects on the γ-aminobutyric acid type A ($GABA_A$), nicotinic acetylcholine (nACh), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptors with no effect on NMDA receptors (Carla and Moroni, 1992; Pistis et al., 1997; Krasowski and Harrison, 1999). Propofol is an anesthetic frequently used in humans for anesthesia induction, maintenance and sedation, which potently modulates the $GABA_A$ and

glycine receptors with little effect on NMDA receptors (Hales and Lambert, 1991; Pistis et al., 1997; Rudolph and Antkowiak, 2004).

The direct modulation of c-Fos expression in olfactory regions by the anesthetic agent itself is undesirable. This is one of the major caveats with ketamine anesthesia (Duncan et al., 1998). Pentobarbital is reported to cause minimal c-Fos activation in most brain regions (Marota et al., 1992; Takayama et al., 1994), whereas propofol has produced mixed findings (Nagata et al., 1998; Kidambi et al., 2010). The effect of these drugs on c-Fos expression within olfactory regions is not well characterized.

We also wanted to determine whether complimentary evidence of information consolidation could be revealed by other types of histological changes. Modulation of Neural Cell Adhesion Molecule (NCAM) polysialylation is thought to play a major role in the synaptic restructuring necessary for memory consolidation (Schachner, 1997). NCAM is a glycoprotein involved in cell-cell adhesion, neurite outgrowth, neurogenesis and synaptic plasticity (Durbec and Cremer, 2001; Bonfanti, 2006). Earlier studies have shown that memory acquisition leads to an increased expression of synaptic membrane glycoproteins (Rose, 1995b, a), and blocking glycoprotein synthesis by intracranial injection of 2-deoxygalactose produces task-specific amnesia (Scholey et al., 1993). Similarly, intracranial injection of an anti-NCAM antibody at 6 hours post-training produces amnesia for passive avoidance (Scholey et al., 1993; Alexinsky et al., 1997) and odor-reward association tasks (Roullet et al., 1997).

Memory acquisition has been shown to involve a transient increase in hippocampal PSA-NCAM, specifically at 12 hours post-training in numerous tasks including odor-reward association (Foley et al., 2003), passive avoidance (Doyle et al., 1992; Fox et al., 1995), spatial learning (Murphy et al., 1996) and fear conditioning tasks (Sandi et al., 2003). These effects

occur within the neurogenic region of the hippocampus; however, they are reported to function independently of changes in neurogenesis (Fox et al., 1995; Foley et al., 2008). Drugs that produce memory-enhancing effects have also been shown to augment NCAM polysialylation within the dentate gyrus (Murphy et al., 2006; Foley et al., 2008; Foley et al., 2009). Interestingly, anesthesia-induced amnesia prevents the transient increase in hippocampal PSA-NCAM, yet nootropic drugs that reverse amnesia, also restore expression of PSA-NCAM (O'Gorman et al., 1998). The diversity of tasks assessed and effects of memory-enhancing drugs suggest that PSA-NCAM may be a common mechanism of memory consolidation.

The hippocampus, however, is not involved in simple olfactory recognition memory (Staubli et al., 1995; Bunsey and Eichenbaum, 1996) and odorant treatment alone has no effect on hippocampal PSA-NCAM (Foley et al., 2003). The role of NCAM in memory consolidation outside of the hippocampus is not well understood. PSA-NCAM-positive cells have been reported to occur within the piriform cortex and PSA-NCAM is proposed to play an important role in olfactory learning in this region (Knafo et al., 2005). We therefore assessed whether odor recognition memory involves changes in PSA-NCAM expression by quantifying the PSA-NCAM labeling within the APC and OFC 12 hours after presentation of a novel odorant mixture. We also assessed whether similar changes could occur under anesthesia and to what extent.

Another potential means of information consolidation could be reflected through changes in the proliferation and survival rates of new adult-born neurons. Neurogenesis continues to occur throughout adulthood in select regions of the vertebrate brain. The subventricular zone is one of two undisputed neurogenic regions that gives rise to neural progenitor cells (Lois and Alvarez-Buylla, 1994; Gould, 2007). These cells enter the rostral migratory stream (RMS) and travel to the olfactory bulb in the anterior-most part of the brain where the precursor cells can

differentiate into the interneurons of the glomerular and granule cell layers (Lledo et al., 2006). A number of these cells will integrate into the local circuitry of the bulb (Carlen et al., 2002; Carleton et al., 2003); however, a large percentage fail to establish appropriate connectivity and die off (Biebl et al., 2000; Petreanu and Alvarez-Buylla, 2002). The subgranular zone of the hippocampus is another neurogenic region, although the progenitors in this region only migrate a short distance giving rise to the granule cells of the dentate gyrus (Ehninger and Kempermann, 2008). The process of adult neurogenesis is thought to occur in order to maintain and refine hippocampal and bulbar networks (Imayoshi et al., 2008).

It has been shown that odor enrichment can produce enhanced olfactory bulb neurogenesis and improved olfactory memroy (Rosselli-Austin and Williams, 1990; Rochefort et al., 2002; Rochefort and Lledo, 2005). We therefore wanted to determine if odorant exposure during periods of anesthesia was able to produce a similar enhancement of neurogenesis, that is, we wanted to assess whether conscious perception was required for odor-induced neurogenesis. We analyzed the proliferation and migration of newborn neurons by quantifying the number of PSA-NCAM-positive cells within the rostral migratory stream. We also analyzed the survival of newborn neurons through BrdU (5-bromo-2'-deoxyuridine) labeling. BrdU is an analogue of the nucleoside thymidine that is incorporated into dividing cells during S-phase, thus allowing one to label, quantify and track newborn neurons (del Rio and Soriano, 1989).

Our current work suggests that consolidation in olfactory regions during anesthesia may not be a universal trait amongst all anesthetics; however, these data are not conclusive. Furthermore, previous findings of c-Fos-mediated consolidation during anesthesia are corroborated by changes in neurogenic proliferation, but not survival. NCAM polysialylation in

olfactory-associated cortical regions was not augmented during the acquisition of odor recognition memories, thus the effects of anesthesia could not be determined.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Animals

For all experiments, 7-week old male Sprague Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Animals were maintained in a controlled temperature (20-22ºC) and humidity environment with a constant 12:12 light-dark cycle (on 07:00 - 19:00 hr). Animals were individually housed to minimize incidental odors and allowed one week to acclimate to the colony and odor environment prior to treatment. The environmental odors in the housing facility were also strictly controlled to minimize any variation. Food and water were available *ad libitum*. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh.

4.2.2 Preliminary Experiment: Screening of Anesthetic Agents

Table 4-1. Doses of various anesthetics

Anesthetic	*AVG Anesthetic Dose (mg/kg)*	*n =*
Pentobarbital	128.1 ± 10.8	5
Etomidate	44.1 ± 3.0	2
Fentanyl / Medetomidine	0.329 ± 0.042 / 0.174 ± 0.022	3
Ketamine / Xylazine	138.4 ± 11.6 / 18.45 ± 1.55	3
Fentanyl	0.410 ± 0.009	2
Medetomidine	1.629	1
Saline	n/a	2
		Total = 18

Each rat was handled and given a saline injection (0.3ml) once a day for two days. On the third day rats were anesthetized and sacrificed two hours after reaching a surgical depth of anesthesia (absence of response to repeated foot pinch). The anesthetics tested were pentobarbital (Nembutal, Ovation Pharmaceuticals, Deerfield, IL), etomidate (Amidate, Hospira, Lake Forest, IL) and Fentanyl/Medetomidine (Fentanyl, Hospira, Lake Forest, IL / Domitor, Pfizer, New York, NY). Ketamine (Ketaset, Fort Dodge, Fort Dodge, IA) and xylazine (Anased, Lloyd Laboratories, Shenandoah, IA) anesthesia was also used as a positive control. Fentanyl alone and medetomidine alone were also tested, but did not produce robust anesthesia. All anesthetics were delievered via intraperitoneal injection. Doses for each group are listed in table 4-1. Lamps and heating pads were utilized to warm the animal as described in experiment 'A'; however, direct measure of body temperature was not assessed. An additional control group received only saline injection (1.3ml). A total of 18 animals were used for anesthetic screening.

4.2.3 Experiment E: Pentobarbital Anesthesia

Table 4-2. Group Layout: Experiment 'E'

Group	*Condition (TEST day)*	*Anesthetic Dose (mg/kg Pento)*	*n =*
E1	Pentobarbital & Novel Odor	111.3 ± 1.8	9
E2	Pentobarbital Alone	118.6 ± 9.6	(8) 5
			Total = (17) 14

Rats received one day of handling, then given a saline injection (0.3 ml, *i.p.*) for 4 days and handled in the hard-ducted hood where testing would take place to reduce c-Fos activation resulting from the injection or environmental factors. On the test day, rats were fully anesthetized to a surgical depth with pentobarbital and placed in the hard-ducted hood for twenty minutes of odorant exposure. A tube containing 35µl of the undiluted odorant mixture was placed approximately 1-inch from the snout in a typical rat cage during the exposure period, after which the tube was removed and the animal allowed to air off for 15 minutes. The odorant mixture used was the same combination used in experiments 'A', 'B' and 'D'. It was composed of an equal mixture of 6 individual odorants (obtained from Sigma, St. Louis, MO): isoamyl acetate, cineole, octanol, decanal, capronaldehyde, and *R*-(-)-carvone. Animals were sacrificed by pentobarbital overdose 2 hours after exposure to the odorant mixture, or a comparable time point in non-exposed animals. An average dose of 114.9 ± 3.0 mg/kg pentobarbital anesthesia was administered in experiment 'E' over an approximate 40 minute period. There were no significant differences for anesthetic dose between groups ($p = 0.34$; student's t-test; Table 4-2). Lamps and heating pads were utilized to warm the animal as described in experiment 'A'; however, direct measure of body temperature was not assessed. A total of 17 animals were used in this experiment. Three animals in group E2 died prematurely due to anesthetic treatment.

4.2.4 Experiment F: Propofol Anesthesia

Table 4-3. Group Layout: Experiment 'F'

Group	*Condition (TEST day)*	*Cumulative Anesthetic Dose (mg/kg Propofol)*	*n* =
F1	Propofol & Novel Odor	436.4 ± 14.3	(9) 8
F2	Propofol Alone	428.0 ± 24.0	8
			Total = (17) 16

In experiment 'F', rats received one day of handling and 3 days of saline injections (0.3 ml, *i.p.*) prior to the test day. During testing, rats were given an initial dose of 200 mg/kg propofol (Propoflo; Abbott Laboratories, North Chicago, IL) through intraperitoneal injection. They were given a follow-up dose of 200 mg/kg after 35 minutes, and a small booster of variable size was given as necessary, to reach a surgical plane of anesthesia, after 70 minutes. Rats receiving the odorants were exposed at 85-95 minutes following the initial propofol injection. This dosing regimen was found to produce reliable and prolonged loss-of-righting-reflex via intraperitoneal route without the use of concomitant detergents. Propofol is typically administered through intravenous injection; however, others have shown that intraperitoneal injection can produce robust anesthesia when administered at much higher doses (Irifune et al., 1999). Rats received an average cumulative dose of 432.2 ± 13.5 mg/kg propofol over an approximate 90 minute period. There were no significant differences in anesthetic dose between groups (p = 0.77; student's t-test; Table 4-3). Lamps and heating pads were utilized to warm the animal as described in experiment 'A'; however, direct measure of body temperature was not assessed. The odorant mixture was delivered in a typical rat cage within a hard-ducted hood to control residual odors. Two tubes of the undiluted odorant mixture (35µl/tube) were introduced into the rat cage. The odorant mixture used was composed of an equal mixture of 6 individual odorants

(obtained from Sigma, St. Louis, MO): isoamyl acetate, cineole, octanol, decanal, capronaldehyde, and *R*-(-)-carvone. Animals received twenty minutes of pulsatile odorant exposure to mimic natural odor exploration. A tube was placed approximately 1 inch from the snout for ten seconds at the beginning, middle and end of the twenty minute exposure period. Both tubes were placed approximately 5 inches from the snout for the remainder of the time. Animals were sacrificed two hours after exposure to the odorant mixture or a comparable time-point in animals not receiving the odorants.

4.2.5 Experiment G: PSA-NCAM

Table 4-4. Group Layout: Experiment 'G'

Group	*Condition (TEST day)*	*Anesthetic Dose (mg/kg Ketamine, Xylazine ± S.E.)*	*n* =
G1	Novel Odor	n/a	8
G2	Anesth. & N. Odor	156.1 ± 7.0, 20.9 ± 0.9	8
G3	Anesthesia Alone	150.2 ± 2.5, 20.1 ± 0.3	9
			Total = 25

One week after arrival, animals received one day of handling, then were given a saline injection (0.3 ml, *i.p.*) once a day for the following three days. Testing was performed the next day where animals were exposed to the odorants alone, anesthesia alone, or the odorants while anesthetized. Rats were sacrificed 12 hours after their respective treatments, a time point shown to correspond to stimulus-induced changes in PSA-NCAM (Foley et al., 2003). Exposure to the odorant mixture was performed using a clean, empty rat cage in a hard-ducted hood similar to that

described in experiment 'F' for anesthetized animals. For awake animals, two tubes of the undiluted odorant mixture were placed in the empty cage, and the rat was allowed to freely interact with the tubes for the duration of the 20 minute odorant exposure period. The odorant mixture used was an equal mixture of 6 individual odorants (obtained from Sigma, St. Louis, MO): isoamyl acetate, cineole, octanol, decanal, capronaldehyde, and *R*-(-)-carvone. Animals were anesthetized with ketamine/xylazine anesthesia over an approximate 30 minute period with an average dose of 153.0 ± 3.5 and 20.5 ± 0.5 mg/kg, respectively. There were no significant differences for anesthetic dose between groups ($p = 0.42$; student's t-test; Table 4-4). Lamps and heating pads were utilized to warm the animal as described in experiment 'A'; however, direct measure of body temperature was not assessed.

4.2.6 Experiment H: Neurogenesis

Table 4-5. Group Layout: Experiment 'H'

Group	*Condition*	*Cumulative Anesthetic Dose (mg/kg Ketamine, Medetomidine ± S.E.) Day: 20 / 21 / 22*			*n* =
H1	Odor Only	n/a	n/a	n/a	3
H2	Air Only	n/a	n/a	n/a	6
H3	Odor and Anes	94.1 ± 19.0, 0.63 ± 0.13	85.8 ± 10.7, 0.57 ± 0.07	74.9 ± 0.08, 0.50 ± 0.001	7
H4	Air and Anes	98.2 ± 15.4, 0.65 ± 0.10	75.0 ± 0.03, 0.50 ± 0.00	87.5 ± 7.7, 0.58 ± 0.05	5
					Total = 21

Upon arrival, rats received two consecutive days of BrdU (5-bromo-2'-deoxyuridine, Sigma, St. Louis, MO; Days 1 and 2) intraperitoneal injections (50 mg/kg dissolved in sterile saline). On days 20, 21 and 22, rats received their respective conditions (Table 4-5) for two hours each day.

Rats either remained awake or were anesthetized with ketamine (Ketaset, Fort Dodge, Fort Dodge, IA) and medetomidine (domitor; Pfizer, New York, NY) anesthesia prior to treatment. Anesthetized animals were given an initial dose of 75mg/kg ketamine and 0.5mg/kg medetomidine. Additional booster doses were administered as needed. Cumulative anesthetic for each group is shown in table 4-4. There were no significant differences in the average anesthetic dose between groups ($p = 0.84$; student's t-test).

Animals were placed in an empty rat cage within a hard-ducted hood for administration of respective treatments. Air was pumped (1L/min) through a flask and into the sealed, empty cage. The 125ml flask containing either 50ml of a banana scent (isoamyl acetate, Sigma) or a control, non-odiferous medium (mineral oil, Sigma) and was placed in a water bath at 25°C. Animals exposed to scent received 10 minutes of the odorant, then 10 minutes of air, alternating, throughout the 2 hour exposure period. Animals in the 'Air' condition received air passed over mineral oil for the 2 hour duration. Animals received three consecutive days of their respective treatments and were sacrificed on day 40.

4.2.7 Immunohistochemistry

At the designated time on the testing day animals were sacrificed and perfused with a solution of 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.0). Brains were post-fixed overnight and cryoprotected in a 30% sucrose/PBS solution with 0.01% sodium azide as a preservative. 30µm sections were generated for fluorescent microscopy using a sliding microtome (Leica, Buffalo Grove, IL) equipped with a freezing stage (Physitemp Instruments, Clifton, NJ). Sections were blocked with 10% donkey blocking buffer (PBS with 10% donkey serum and 0.2% Triton X-100) for 2 hours at room temperature. Primary antibodies against c-

Fos (Calbiochem-EMD Biosciences, San Diego, CA; 1:5000), PSA-NCAM (Chemicon-Millipore, Billerica, MA) and/or BrdU (GE Healthcare, Piscataway, NJ) were diluted in donkey blocking buffer and incubated with free-floating sections overnight at 4ºC. Sections were then washed with PBS and incubated with Alexa Fluor 594 and/or 488-conjugated secondary antibodies (Invitrogen, Carlsbad, CA; 1:500) in the dark. Sections were co-stained with the nuclear marker DAPI (4',6-diamidino-2-phenylindole; Sigma), washed and mounted onto slides using fluoromount-G mounting medium (SouthernBiotech, Birmingham, AL).

Brains for BrdU immunohistochemistry (Experiment 'H') were fixed in 10% formalin (Fisher Scientific, Hampton, NH), but embedded in paraffin wax with the help of the core histology facility. 6µm sections were generated by a rotary microtome (American Optical Co., Buffalo, NY) placed in a warm water bath and mounted onto slides. Paraffin sections were prepared for antibody staining by first exposing the slides to a series of hydrating washes (xylene-ethanol-water) followed by PBS. Antigen retrieval was next performed by microwaving the sections in a 10mM sodium citrate solution for 12 minutes. Sections were then processed for fluorescent immunohistochemistry as described above.

4.2.8 Quantification

Staining was imaged using an Olympus IX-81 widefield microscope equipped with a monochrome ORCA-ER (Hamamatsu, Bridgewater, NJ) peltier-cooled CCD camera. Images were acquired and analyzed using QED - In Vivo and Image-Pro Plus (Media Cybernetics, Bethesda, MD) software. Fluorescent images for experiments 'E' and 'F' were acquired under identical settings and uniformly quantified by a threshold method. They were additionally screened by a set size and subjected to a watershed split function. Comparisons were drawn only

between sections stained and imaged in the same session. Sections for fluorescent c-Fos imaging were taken from +3.5mm, +3.3mm and +3.1mm rostrocaudal bregma in the cortex and +6.8mm and +7.9 rostrocaudal bregma in the olfactory bulb. Fluorescent images for experiments 'G' (PSA-NCAM) and 'H' (BrdU) were also acquired under identical settings, but were quantified manually by blinded observers. PSA-NCAM-labeled sections were taken from +3.3mm and +3.1mm rostrocaudal bregma in the cortex. BrdU-labeled sections were taken from approximately +7.4mm rostrocaudal bregma in the olfactory bulb. Labeling in fluorescent tissue was represented as positive cell number per mm^2.

4.2.9 Statistical Analysis

One-way ANOVA was used to determine a significant overall difference followed by Tukey's *post hoc* analysis for inter-group comparison. A student's t-test was utilized for experiments containing only two groups. Values are described as group means ± standard error. For all analyses, differences were considered significant when $p < 0.05$.

4.3 RESULTS

4.3.1 Absence of consolidation during pentobarbital or propofol anesthesia

We screened a number of injectible anesthetics to find the drug with the lowest degree of anesthetic-induced c-Fos activation (Fig. 4-1). Based on our anesthetic screen, we investigated the effects of pentobarbital as this anesthetic produced little c-Fos immunoreactivity in the

olfactory bulb. This allowed us to address the lack of an effect found in the olfactory bulb with ketamine/xylazine anesthesia. In this previous experiment potential effects were obscured by high levels of anesthetic-induced c-Fos in this region (Fig. 2-7). We could also assess whether the effects previously found in the APC and OFC (Fig. 2-5, 2-6) applied to other anesthetic types.

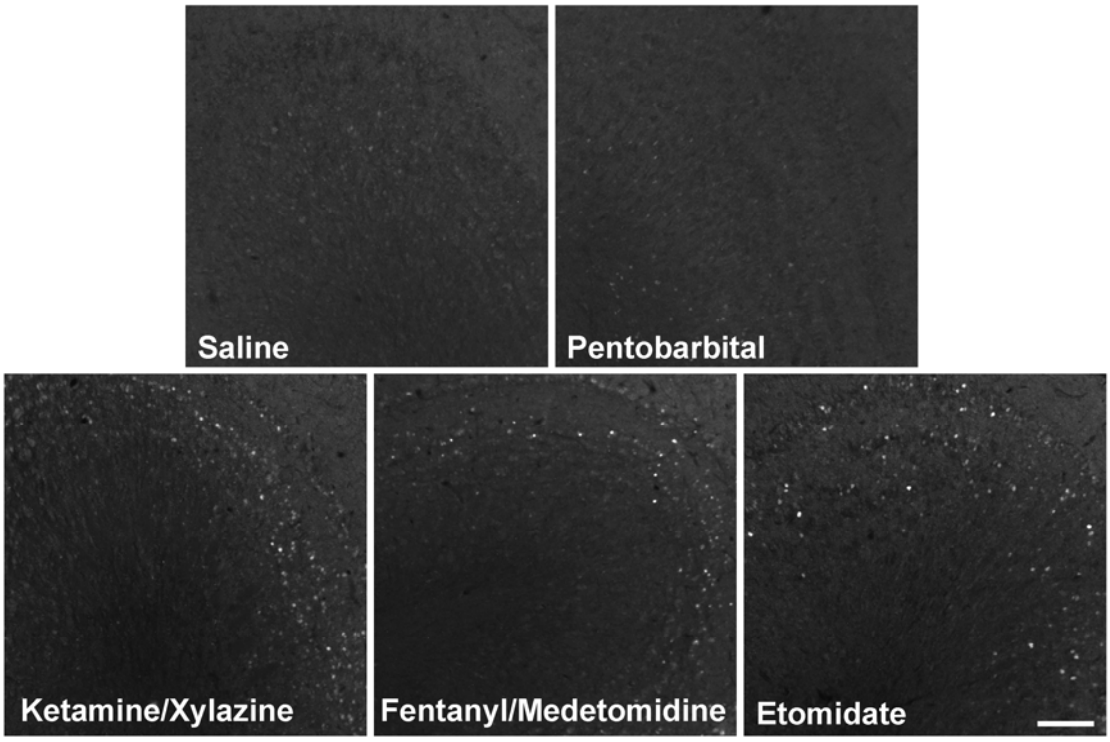

Figure 4-1. Expression of c-Fos in the olfactory bulb with various anesthetics
Representative fluorescent micrographs of c-Fos immunolabeling in the granule and mitral cell layers of the olfactory bulb. Data from anesthesia screen preliminary experiment. Scale bar = 100µm.

We anesthetized rats with pentobarbital (experiment 'E') and delivered either a novel odorant mixture (Pento & N. Odor; group E1) or no odorant mixture (Pento Alone; E2). These two groups were compared and c-Fos expression was quantified in the OB(GCL), APC and OFC

(Fig. 4-2). Student t-tests revealed no significant differences between Pento & N. Odor (E1) and Pento Alone (E2) groups in any region, unlike that observed with ketamine/xylazine anesthesia.

We next investigated whether propofol anesthesia would allow odorant-induced changes in c-Fos similar to ketamine/xylazine (experiment 'F'). Comparison of animals anesthetized with propofol, then given a novel odorant mixture (Propofol & N. Odor; group F1) or anesthetized with no odorant mixture (Propofol Alone; F2) revealed no significant differences between these two groups in any of the regions assessed (student t-test). It is important to note that in our original experiment (experiment 'A') naïve animals demonstrated very low baseline levels of c-Fos expression, unlike that seen in our pentobarbital or propofol alone groups, suggesting that high levels of anesthetic-induced c-Fos may be obscuring potential effects in these groups. Though these data do not indicate an effect with propofol or pentobarbital, we cannot rule out potential effects with these anesthetics.

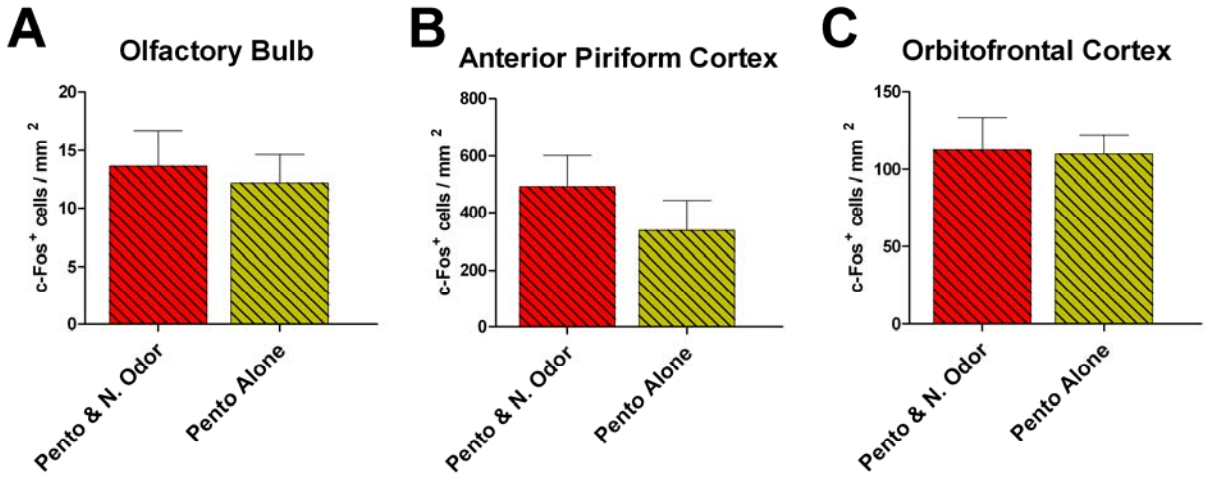

Figure 4-2. Effects of pentobarbital anesthesia on odor-induced c-Fos expression

Quantification of c-Fos immunolabeling in the granule cell layer of the olfactory bulb (A), in the anterior piriform cortex (B) and in the orbitofrontal cortex (C). Data from experiment 'E'.

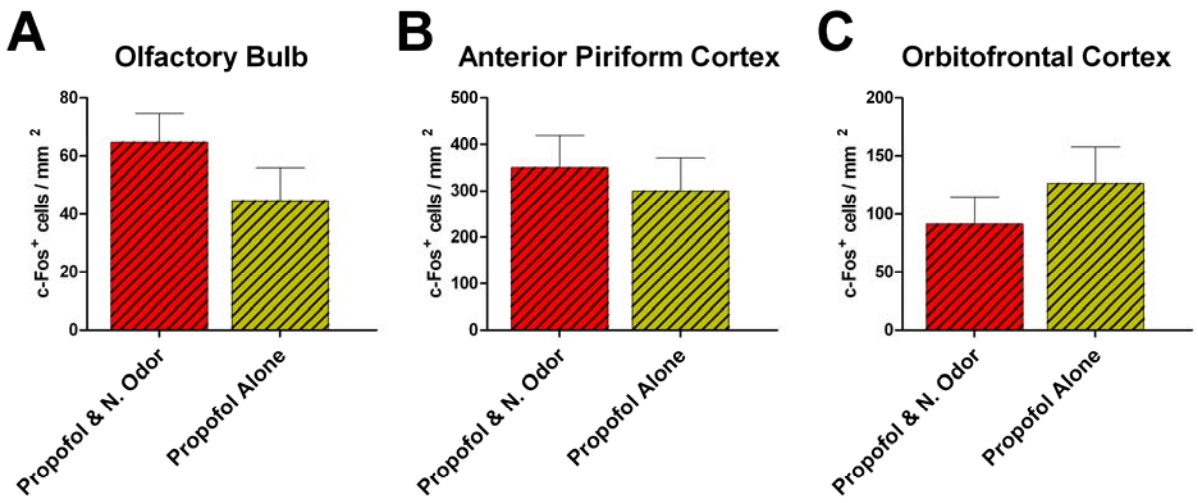

Figure 4-3. Effects of propofol anesthesia on odor-induced c-Fos expression

Analysis of c-Fos protein expression in the granule cell layer of the olfactory bulb (A), in the anterior piriform cortex (B) and in the orbitofrontal cortex (C). Data from experiment 'F'.

4.3.2 Cortical PSA-NCAM expression is unaffected by odorants and anesthesia

Consolidation of associative odor memories has been shown to involve transient upregulation of PSA-NCAM in the hippocampus at twelve hours after the memory event (Foley et al., 2003). We wanted to test whether NCAM polysialylation occurred for simple odor recognition memories in the APC and OFC, and what effect anesthesia might have on this process. Thus, we wanted to determine whether other forms of histological consolidation could occur under ketamine/xylazine anesthesia that complement our c-Fos data (Fig. 2-5, 2-6). Rats were treated with scent and/or anesthesia similar to that in experiment 'A', however rats were sacrificed twelve hours after their respective treatments to assess PSA-NCAM expression (experiment 'G'). We compared rats that received a novel odorant mixture (group G1), with rats that were given

anesthesia and a novel odorant mixture (G2) or anesthesia alone (G3). Naïve animals (A9) were also used as a negative control. PSA-NCAM-positive cells were found in both regions for all groups; however, the density of cells was very sparse. One-way ANOVA of the four groups (Fig. 4-4) revealed no significant differences in the APC ($F_{(3,29)} = 0.46$; $p = 0.71$) or the OFC ($F_{(3,29)} = 1.86$; $p = 0.16$). These data suggest that NCAM polysialylation in the APC and OFC is not affected by simple odor recognition memory at 12 hours following odorant presentation. Therefore, no conclusions can be drawn regarding any parallels between PSA-NCAM and c-Fos related consolidation.

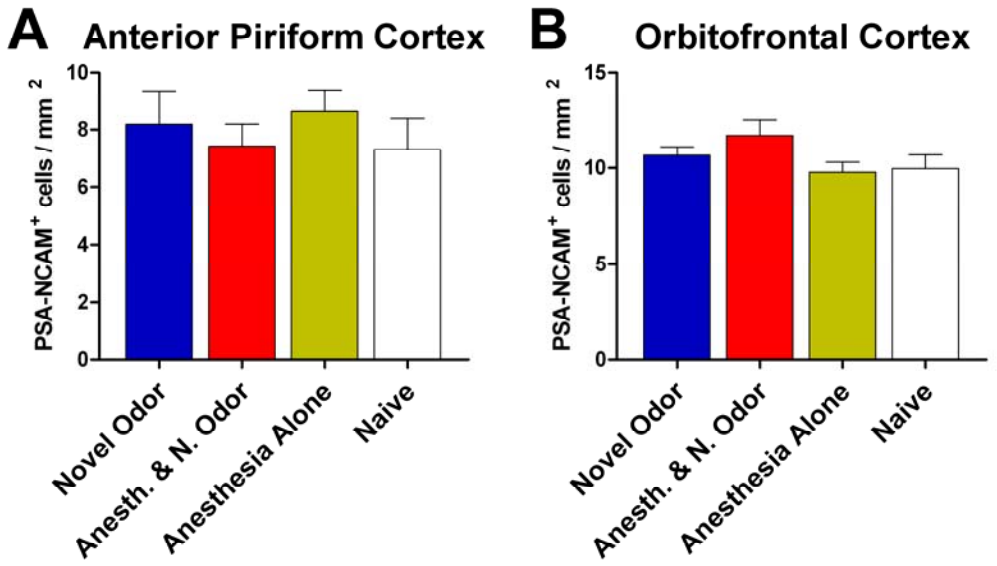

Figure 4-4. Absence of PSA-NCAM effects in the APC and OFC

PSA-NCAM immunolabeling (cells per mm^2) in the anterior piriform cortex (A) and orbitofrontal cortex (B). Data from experiment 'G'.

4.3.3 Effects of odor and anesthesia on neurogenesis

To determine if the proliferation of adult neurogenesis was affected by administration of the odorant mixture in the presence or absence of ketamine/xylazine anesthesia, we analyzed the density of PSA-NCAM positive cells within the rostral migratory stream in experiment 'G' (Fig. 4-5). Naïve animals (group A9) were compared to animals that had received either a novel odorant mixture alone (G1), anesthesia and a novel odorant mixture (G2) or anesthesia alone (G3), twelve hours prior to sacrifice (Fig. 4-5). One-way ANOVA revealed a significant effect of PSA-NCAM density for the section average ($F_{(3,29)} = 8.43$; $p < 0.001$). Pair-wise comparison (tukey test; Fig. 4-5A) of the section average revealed a significant difference between Anesthesia & N. Odor (G2) and Anesthesia Alone groups (G3; $p < 0.05$) as well as between Anesthesia & N. Odor (G2) and Naïve groups (A9; $p < 0.001$). Furthermore, a near-significant trend was found between Novel Odor (G1) and Naïve (A9) groups ($p = 0.06$; tukey posttest).

It is reported that most neuroblasts migrate along the RMS at a rate of approximately 100–120 μm/hr (Murase and Horwitz, 2002; Murase et al., 2008). The speed of migration varies dramatically with a normally distributed migration rate ranging from 20-180 μm/hr (Murase et al., 2008). It is possible that our 20 minute potent odorant exposure paradigm produced a transient bolus of migratory neuroblasts. While it is difficult to determine the exact location of such a migratory bolus, the brain region sampled (+3.1mm and +3.3mm rostrocaudal bregma) is appropriate to see effects at 12 hours post-treatment. As a migratory bolus may produce different effects along the rostrocaudal axis, we also report individual section densities.

For the individual section +3.3mm bregma (Fig. 4-5B) we found a significant group effect ($F_{(3,29)} = 6.66$; $p < 0.01$; one-way ANOVA). Significant pair-wise effects were found between Novel Odor (G1) and Naïve (A9; $p < 0.05$) as well as Anesthesia & N. Odor (G2) and

Naïve (A9; $p < 0.01$) groups. A near-significant trend was also found between Anesthesia & N. Odor (G2) and Anesthesia Alone groups in this region ($p = 0.057$; tukey posttest).

At section +3.1mm bregma (Fig. 4-5C), a significant group effect was also found ($F_{(3,29)} = 4.53$; $p < 0.05$; one-way ANOVA). *Post-hoc* comparison revealed that Anesthesia & N. Odor (G2) and Naïve (A9) groups were significantly different ($p < 0.01$); however, there were no other significant effects. The Anesthesia & N. Odor group (G2) was not significantly different than the Anesthesia Alone group (G3) in this section; however, a trend was again found ($p = 0.06$). Taken together, these data suggest that a novel odorant mixture induces enhanced neuroblast proliferation and migration in the presence or absence of anesthesia. The presence of both the odorant mixture and anesthesia appeared to produce the most robust effect, suggesting that ketamine/xylazine anesthesia, itself, enhanced proliferation. Indeed, ketamine has been reported to enhance neurogenesis (Keilhoff et al., 2004) and be neuroprotective (Hudetz and Pagel, 2010). Most importantly, however, the pattern of consolidation as indicated by proliferation between Anesthesia & N. Odor (G2) and Anesthesia Alone (G3) groups mimics that found for c-Fos consolidation in previous experiments (Fig. 4-5A vs Fig. 2-5 and 2-6).

In another study (experiment 'H') we investigated the survival of adult newborn neurons following odorant exposure with and without simultaneous anesthesia. We analyzed the number of BrdU-labeled cells residing within the olfactory bulb 20 days after odorant and/or anesthesia treatment (40 days after initial BrdU injection; Fig. 4-6). Comparison of animals treated with Odor Alone (group H1), Air Alone (H2), Odor & Anes (H3) and Air & Anes (H4) revealed no significant group effects (One-way ANOVA; $F_{(3,17)} = 0.12$; $p = 0.95$). This suggests that our method of olfactory stimulation was not sufficient to enhance the survival of newborn neurons within the olfactory bulb, thus no conclusions could be drawn for the effects of anesthesia.

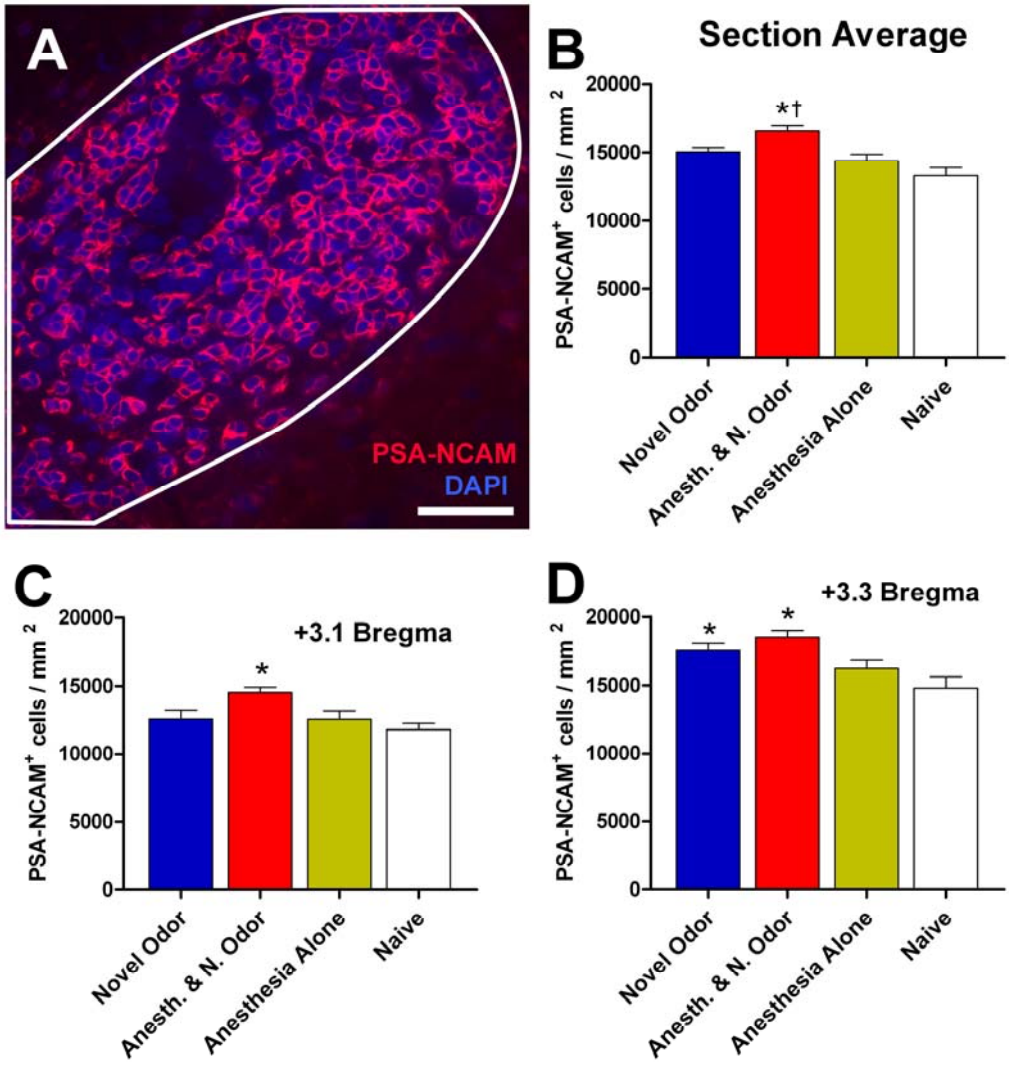

Figure 4-5. Odor-induced precursor proliferation in the RMS with and without anesthesia

Representative fluorescent micrograph of PSA-NCAM immunolabeling (red) and DAPI nuclear stain (blue) in the rostral migratory stream (outlined in white; A). Quantification of PSA-NCAM density for the section average (B) and for individual sections at +3.1 mm (C) and +3.3 mm (D) rostrocaudal bregma. Data from experiment 'G'. (*) $p < 0.05$ relative to 'Naïve', (†) $p < 0.05$ relative to 'Anesthesia Alone', one-way ANOVA with Tukey *post hoc* test. Scale bar = 30µm.

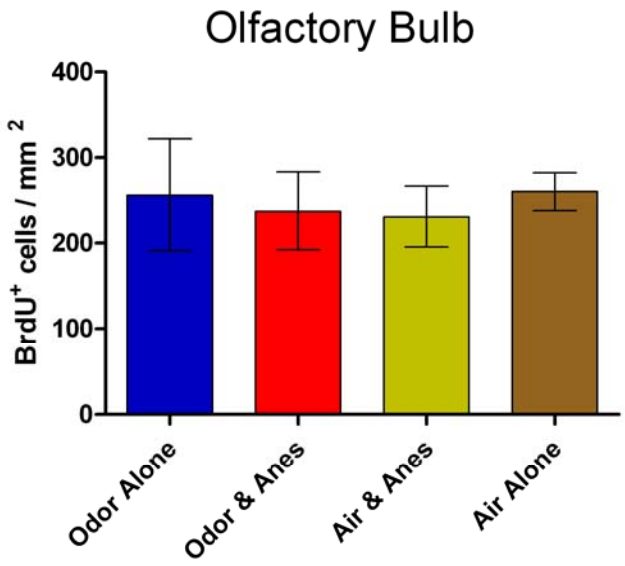

Figure 4-6. No effect on newborn neuron survival with odorant and/or anesthesia exposure

Effects of odorant exposure and anesthesia on BrdU-labeled neurons in the granule cell layer of the olfactory bulb. Data from experiment 'H'.

4.4 DISCUSSION

In our earlier experiments ('A' and 'B'), we found changes in c-Fos expression during and after ketamine/xylazine anesthesia, which may have indicated some level of information consolidation. We therefore wanted to assess whether this effect was common to all anesthetics. Based on our preliminary work, we anesthetized animals with pentobarbital and found no impact of subsequent odorant exposure on c-Fos histology. Comparable results were found with the anesthetic propofol. However, similar to that seen with ketamine/xylazine anesthesia in the olfactory bulb (Fig. 2-7), it is possible that anesthetic-induced c-Fos is obscuring potential effects with pentobarbital and propofol. Thus, the extent of stimulus-induced c-Fos with various

anesthetics remains inconclusive. Regardless of the anesthetic ubiquity, the observation of odor-induced information registration with even a single anesthetic is a considerable finding.

Most common anesthetics are thought to act through the potentiation of inhibitory transmembrane channels such as $GABA_A$ and glycine (Rudolph and Antkowiak, 2004). Ketamine, in contrast, has little effect on these inhibitory receptors, but is instead thought to act through the inhibition of NMDA, and to a lesser extent, nACh signaling (Harrison and Simmonds, 1985; Martin and Lodge, 1985; Carla and Moroni, 1992; Krasowski and Harrison, 1999). The unique mechanism of action for ketamine anesthesia may explain the unique permissive effects found with c-Fos-mediated consolidation during ketamine/xylazine anesthesia in our studies. The gaseous anesthetics nitrous oxide and xenon also have powerful effects on the NMDA and nACh receptors with comparatively little effect on the $GABA_A$ and glycine receptors relative to other volatile anesthetics (Yamakura and Harris, 2000; Hirota, 2006; Franks, 2008). The mechanisms of these anesthetics involve a number of key similarities with that of ketamine; therefore, it is possible that follow-up studies with nitrous oxide or xenon may reveal a permissive effect with c-Fos consolidation similar to that of ketamine/xylazine.

During the screening of anesthetics, it was observed that animals anesthetized with etomidate were still responsive to a sudden, loud acoustic stimulus (*e.g.* a hand clap) despite a very high dose of anesthesia. Etomidate is a short acting anesthetic with a potent effect on $GABA_A$ receptor function (Yang and Uchida, 1996; Pistis et al., 1997). The acoustic response occurred reproducibly despite all physiological signs indicating full anesthesia. This effect was not found in pentobarbital, propofol or even ketamine/xylazine treated animals. Based on the residual processing in these etomidate-anesthetized animals, changes in c-Fos might also occur with olfactory stimuli, similar to that seen with ketamine/xylazine. We purposefully avoided the

use of volatile anesthetics in our current olfactory experiments due to associated odors and the necessary involvement of olfaction/respiration in drug administration, a clear confounding variable.

In our current study we confirmed the presence of PSA-NCAM positive cells within the piriform and orbitofrontal cortices; however, simple odor recognition memory did not appear to produce any changes in the number of PSA-NCAM-expressing cells within these regions. PSA-NCAM is suspected to play an important role in the hippocampus as disruption of NCAM polysialylation attenuates induction of long-term potentiation (LTP) in hippocampal slices and produces spatial learning difficulties on the Morris water maze task (Becker et al., 1996; Muller et al., 1996), with similar results found in NCAM knockouts (Cremer et al., 1994; Muller et al., 1996). It has been shown that hippocampal-dependent spatial learning and memory abilities correlate to NCAM PSA levels (Sandi et al., 2004). Indeed, the changes in PSA-NCAM expression found previously were located in the dentate gyrus of the hippocampus (Fox et al., 1995; Foley et al., 2003), which is a region of adult neurogenesis (Gould, 2007). Based on BrdU labeling, it is reported that these effects were not dependent on neurogenesis (Fox et al., 1995; Foley et al., 2008). However, PSA-NCAM is expressed only in neural precursors and is down-regulated once neurons mature (Encinas et al., 2006). Though no effects were found with PSA-NCAM in the APC or OFC, we did find changes in PSA-NCAM within the rostral migratory stream, a neurogenic region (Gould, 2007). This suggests that neurogenesis, or a permissive neurogenic environment, may somehow be necessary for memory-related changes in PSA-NCAM. Alternatively, PSA-NCAM may, in fact, be involved in the memory processes within the piriform and orbitofrontal cortices; however, changes in these regions may occur on a different timescale relative to that which occurs in the hippocampus (12 hours post-training). It

is also possible that changes in NCAM-polysialylation are only involved in hippocampal-dependent memory processes. Analysis of APC/OFC PSA-NCAM at various time-points following odorant exposure may help clarify this ambiguity.

With respect to neurogenesis, we found effects in proliferation as evident by changes in PSA-NCAM positive cells within the rostral migratory stream; however, no effects were found in survival as evident by BrdU labeling within the olfactory bulb. While much is known about the factors regulating neurogenesis (Lledo et al., 2006; Hagg, 2007), the mechanisms of odor-induced neurogenesis are not well understood. It can be assumed that changes in the brain are occurring due to an interaction of the brain and environment: an interaction that may be disrupted by general anesthesia. However, greater proliferation found in regions of the rostral migratory stream following exposure to the odorant mixture during anesthesia suggests that these processes can still occur, much in the same way that c-Fos processes continue to operate during general anesthesia. The subventricular zone (SVZ) is a centrally located brain region that lies away from the olfactory bulb, yet gives rise to adult born olfactory bulb interneurons (Lois and Alvarez-Buylla, 1994; Gould, 2007). Lesions that detach the olfactory bulb from the forebrain reduce the proliferation rate in the SVZ (Jankovski et al., 1998), suggesting that information from the olfactory bulb is transmitted to the subventricular zone which is used to modulate proliferation rate. The odor-induced proliferation found in our current study supports this idea. This coherent OB/SVZ inter-region communication appears to occur despite surgical doses of ketamine/xylazine anesthesia. In combination with our c-Fos results, these data support the notion of residual inter-region communication and information registration during ketamine/xylazine anesthesia.

Effects found with proliferation, but not survival, suggest that newborn neurons, produced as a result of odor enrichment, do not integrate into the olfactory bulb on arrival. This is not surprising as the majority of neuroblasts arriving from the sub-ventricular zone eventually die off after reaching the olfactory bulb (Biebl et al., 2000; Winner et al., 2002). [^{3}H]thymidine labeling experiments in rodents reveal that approximately 50% of new OB granule cells die off after 45 days, and this number increases to 75% at 12 (Petreanu and Alvarez-Buylla, 2002) and 90% at 21 months (Kaplan et al., 1985). Petreanu and Alvarez-Buylla (2002) found that after 4 consecutive daily [^{3}H]thymidine treatments, a maximum of 2.2% of total granule cell neurons were labeled 15 days later and only 1.5% were labeled at 45 days. Thus, a small transient change in neurogenesis resulting from brief odorant exposure may be visible in the RMS, a highly concentrated region of neural precursors; however, this small change could easily be diluted out in the vast olfactory bulb, especially when viewed on a protracted timescale.

It has been previously shown that complex odor enrichment enhances olfactory bulb neurogenesis (Rosselli-Austin and Williams, 1990; Rochefort et al., 2002; Rochefort and Lledo, 2005). The authors found that enrichment enhanced interneuron survival, but not proliferation, the opposite of what was found in our current study. The discrepancy may depend on the method of assessing proliferation and survival. For proliferation, the authors exposed mice to 20 days of odor enrichment then delivered a single BrdU injection and assessed labeling 4 hours later in the rostral and caudal regions of the subventricular zone and in the caudal RMS. In contrast, we kept rats in a relatively odor deprived environment up until they received a potent 20 minute complex odor stimulus, and then assessed the density of immature neurons 12 hours later within various regions of the RMS as determined by PSA-NCAM immunolabeling. It is possible that our abrupt and potent change in the odor environment was a more effective means

of altering proliferation rate. It should be noted that greater BrdU labeling was indeed found by the authors in the anterior subventricular zone and caudal RMS; however, this effect was not significant (Rochefort et al., 2002). For newborn neuron survival, Rochefort et al. (2002) exposed the animals to forty consecutive days of various odorants for 24 hours a day, whereas our animals were only exposed to three days of a single odorant for 2 hours a day. Insufficient cumulative odorant exposure may be an alternate explanation for the lack of effects on survival in our study.

These data have shown that c-Fos related information consolidation may not occur for every anesthetic. Furthermore, while the extent of information consolidation with ketamine/xylazine anesthesia remains unclear, we have shown that this finding is not unique to c-Fos processes.

5.0 GENERAL DISCUSSION

5.1 POSSIBLE INTERPRETATIONS

We have found that some level of memory-related information consolidation occurs in various brain regions including higher cortical areas despite a surgical plane of ketamine/xylazine general anesthesia. The odorant-induced activation of c-Fos does not indicate that consolidation occurred to completion, but merely that consolidation mechanisms were activated. Our effects found in approach latency suggest that memory formation may still occur, albeit in subconscious pathways. These data indicate a functional disconnect between receiving and perceiving information. However, if storage is occurring, why is there no explicit memory of the event? While the obvious answer may be that complete storage is not actually occurring, there are a number of possibilities. One possibility is that a fully intact memory is formed under general anesthesia, but the percept of the odor has changed. For example, a 'banana' odorant received during anesthesia might be remembered as 'grape', and when presented with 'banana' during the recall test the odor does not correspond to the memory and is reported as novel. This might explain the discrepancy between c-Fos immunoreactivity and behavioral reports (Fig. 2-8). In this view, anesthesia would skew the conscious percept similar to light diffracting through water. The shift in perception for each stimulus would have to vary randomly otherwise anesthesia would simply produce something similar to a coherent negative image of our world. Such a

deficit in stimulus sorting and categorization may be more likely to mediate the anesthetic-induced unconsciousness for anesthetics such as ketamine that augment regional brain activity (Duncan et al., 1998; Langsjo et al., 2005).

It is also possible that conscious perception does not reflect the total information that resides in the brain. This concept is certainly not new and is evident in anyone who cannot recall the date of a loved one's birthday, or a particular piece of trivia that they typically can remember. With respect to general anesthesia, this would suggest that consolidation may take place, but functional recall is absent. This view is supported by numerous clinical findings that have shown that information presented during general anesthesia is stored as evident by subconscious, implicit memory following anesthesia. In one example, thirty patients were presented a story through headphones during surgical propofol/alfentanil/N_2O anesthesia, then asked to read the same story later while awake. These patients showed a significantly faster reading rate (by approximately 40ms per word) of the story presented during anesthesia relative to a new story, similar to that found in control, unanesthetized patients (Munte et al., 1999). Despite some claims to the contrary (Munte et al., 2000; Kerssens et al., 2005), a large body of literature has revealed that implicit, but not explicit, memory can form during a wide range of general anesthetics (Bailey and Jones, 1997; Kiviniemi, 1997; Ghoneim et al., 1999; Lubke et al., 1999; Ghoneim et al., 2000; Iselin-Chaves et al., 2005).

The possibility of subconscious information consolidation is also supported by the blindsight literature, where patients can accurately describe visual stimuli in the absence of a conscious percept (Weiskrantz, 1996; Cowey, 2010). Similar to that found with anesthesia, fully amnestic patients are able to perform normally on measures of implicit memory even though conscious recall is lacking (Warrington and Weiskrantz, 1970; Brooks and Baddeley, 1976;

Squire et al., 1987). These studies indicate that information is received and stored despite amnesia, and suggest that the absence of memory may be due to a failure of recall. However, evidence of implicit memory does not necessarily indicate that explicit memory was stored as well.

Another possible conclusion is that conscious (explicit) and unconscious (implicit) pathways occur as parallel and separate memory systems. Thus, the anesthetized state may prevent consolidation in the explicit memory pathway, while still allowing implicit memory consolidation. Preserved implicit memory in amnesic patients with temporal lobe damage has lead researchers to believe that different neural structures underlie explicit and implicit memory (Squire, 1992; Tranel et al., 1994; Voss and Paller, 2008), therefore activation of only a single pathway may be possible. This conclusion assumes that information consolidation does not occur in explicit memory systems. While the exact roles of the anterior piriform cortex and orbitofrontal cortex in explicit versus implicit memory are not well understood, the c-Fos immunolabeling observed in these regions under anesthesia could indicate that we are actually observing mechanisms of implicit memory consolidation. This would be consistent with the implicit memory effects found in our study and in the aforementioned clinical work.

Alternatively, it is possible that our c-Fos labeling does in fact represent consolidation of explicit memory; however, these processes may have been incomplete or fragmented thus preventing explicit memory recall. Expression of c-Fos is involved in a large cascade of cellular events and anesthesia may have an effect on downstream proteins in this cellular cascade, thus preventing the actions of c-Fos without any observable effect on c-Fos expression itself. As anesthetics can differentially affect various brain regions, it is also possible that quasi normal

consolidation occurred in the APC and OFC; however, the encoding of a retrievable memory required additional regions that were not functioning properly during anesthesia.

Indeed, it is likely that countless molecular events need to occur in numerous coordinated brain regions for an explicit memory to be fully consolidated and recallable. Furthermore, it is plausible that even a slight disruption in any of these components might preclude robust consolidation. Focal lesions to various cortical, limbic and thalamic sites all produce some disruption of olfactory memory (Staubli et al., 1984; Staubli et al., 1986; Staubli et al., 1987a; Slotnick and Risser, 1990; Staubli et al., 1995; Bunsey and Eichenbaum, 1996; Tait and Brown, 2007). Also mechanistically different neural insults, ranging from electroconvulsive shock and hypothermia, to ethanol and anticholinergics, all impair memory (Devietti and Hopfer, 1974; Jensen et al., 1975; Carballo-Marquez et al., 2007; Spinetta et al., 2008). This indeed demonstrates that diverse mechanisms in numerous regions contribute to normal memory consolidation. Thus, the mere activation of certain memory-related proteins provides little evidence of whether or not an explicit memory was formed. Nevertheless, the finding that event-specific registration can still occur, despite a behaviorally unconscious state, indicates a profound discovery.

5.2 CURRENT VIEWS

It has been proposed that immediate early genes, nicknamed “memory proteins”, are essential for the formation of long-term memories and that the amnestic effects of anesthesia are mediated by the disruption of these genes, specifically at the hippocampus (Alkire and Guzowski, 2008). This is based on a study where amnestic doses of propofol anesthesia were shown to block

activity-regulated cytoskeletal (Arc) protein expression in the hippocampus of rats trained on an inhibitory avoidance task. Manipulations that rescued Arc expression in this region also rescued the memory (Ren et al., 2008). Sevoflurane and desflurane have also been reported to block hippocampal Arc expression (Alkire and Guzowski, 2008). Recently, the work from another group suggests that the residual effects of ketamine on memory may be mediated by suppression of c-Fos protein in the hippocampus (Peng et al., 2011).

Arc and c-Fos are induced by learning and display strikingly similar expression patterns in the hippocampus (Grimm et al., 1997; Guzowski et al., 2001; Matsuo et al., 2009; Clark et al., 2011). Knockout of the alpha-isoform of calcium/calmodulin-dependent protein kinase II (alpha-CaMKII) produces deficits in memory function and also eliminates the learning-induced activation of both hippocampal c-Fos and Arc expression (Matsuo et al., 2009). Furthermore, intracranial injections of drugs that enhance or inhibit memory function produce elevated or attenuated Arc protein expression, respectively, in the dorsal hippocampus. Injection of Arc antisense oligodeoxynucleotides into this region of the hippocampus prior to training produces memory impairments on an inhibitory avoidance task (McIntyre et al., 2005). This same procedure has also been shown to impair the maintenance phase of long-term potentiation (LTP) and produce deficits in long-term memory for a spatial water-maze task (Guzowski et al., 2000). Similar behavioral effects were also seen with hippocampal *c-fos* knockdown (Grimm et al., 1997). These studies suggest that the disruption of immediate early genes, specifically c-Fos and Arc, in the hippocampus may underlie amnestic effects.

In the olfactory system, *c-fos* and *arc* also show similar expression patterns. Both genes are activated robustly by a novel odorant and show an attenuated expression profile with a familiar odorant (Montag-Sallaz and Buonviso, 2002). However, consolidation of olfactory

memories does not require hippocampal c-Fos expression (Bunsey and Eichenbaum, 1996; Tronel and Sara, 2002; Roullet et al., 2005b). Olfactory memory tasks appear to activate c-Fos in the hippocampus only if these tasks involve a spatial component, social cues or complex learning (Staubli et al., 1995; Countryman et al., 2005; Roullet et al., 2005a). Spatial olfactory tasks produce elevated c-Fos immunoreactivity only in the CA1, and not the CA3 or dentate gyrus, after learning (Roullet et al., 2005a), which may result from the role of the hippocampus in spatial memory. Olfactory memory tasks that do not involve spatial learning or social cues, produce little observable c-Fos immunoreactivity in the hippocampus (Tronel and Sara, 2002).

It is possible that olfactory memories involve changes in Arc, but not c-Fos, expression in the hippocampus. Despite their similar expression profiles, these genes have different functional roles. c-Fos is a regulatory transcription factor (RTF) and influences neural function by its effects on downstream genes, whereas Arc is an 'effector' protein that directly affects cellular functions, specifically cytoskeletal proteins in a process that is thought to impact neural plasticity (Guzowski, 2002; Terleph and Tremere, 2006). We were unable to find evidence of Arc protein expression in the olfactory bulb with several different antibodies (data not shown) at the time-point used for c-Fos protein expression (2 hours after stimulus), but we did not assess Arc expression in higher cortical olfactory regions or in the hippocampus. Given the similar ability of both genes to be induced by a variety of tasks in the hippocampus (Guzowski et al., 2001; Matsuo et al., 2009; Clark et al., 2011), it is unlikely that these genes would show an opposite pattern of expression for tasks involving olfactory memory. It is therefore plausible that immediate early gene expression in other brain regions is involved in the anesthetic-induced amnesia for olfactory memory. Such a conclusion is supported by lesion studies which have demonstrated that complete ablation of the hippocampus does not prevent the acquisition of odor

associations; although, more complex forms of learning such as odor transitivity and symmetry are impaired (Staubli et al., 1995; Bunsey and Eichenbaum, 1996).

It should be noted that the olfactory system is unique in that it is the only modality that is not necessarily gated through the thalamus. Olfactory processing involves direct connections to the orbitofrontal cortex from the pirifrom cortex, and indirect connections that are sent through the thalamus (Shepherd, 2007). In addition, there are fewer synaptic connections required for olfactory information to reach higher cortical areas, relative to other sensory systems. Thalamocortical projections may also not be necessary for the acquisition of olfactory memories (Staubli et al., 1987a). This unique circuitry may explain the lack of c-Fos activation in the hippocampus (Tronel and Sara, 2002), and suggests the possibility that other loci more central to olfactory pathways may have co-opted this function. One possible region is the anterior piriform cortex, as this area has been implicated to play a role in learning and memory (Barkai and Saar, 2001; Sanchez-Andrade et al., 2005).

It was found that intrahippocampal injection of a *c-fos* antisense oligonucleotide reduced gene expression within the hippocampus, but also in other limbic and cortical regions (Grimm et al., 1997), suggesting that the effects of knockdown on non-olfactory memories could have actually occurred at a location distal to the hippocampus. In support of this, *c-fos* knockdown in regions outside the hippocampus has been shown to impair memory consolidation for conditioned fear, passive avoidance and taste aversion tasks (Lamprecht and Dudai, 1996; Mileusnic et al., 1996; Morrow et al., 1999; Yasoshima et al., 2006). Thus, the hippocampus is not the only critical locus for IEG functions. This is true for both olfactory and non-olfactory memory tasks.

Consistent with the views of Alkire and Guzowski (2008) and others (Ren et al., 2008), we have found that propofol and pentobarbital anesthesia appeared to eliminate learning-induced elevations in IEG expression, albeit in olfactory regions. However, with ketamine/xylazine anesthesia we found that immediate early gene expression not only persisted, but showed changes similar to that which occurred in the awake state. The easiest way to reconcile this finding is that our results were not in the hippocampus, and the hypothesis put forth by Alkire and Guzowski (2008) did not indicate any expectation of a global IEG effect. Little is known about the effects of ketamine/xylazine anesthesia on Arc expression. However, based on our findings, it is likely that ketamine/xylazine anesthesia would not prevent hippocampal Arc activation in memory tasks of another modality. Such a finding would indeed be inconsistent with this model of IEG function and anesthetic-induced amnesia, and would force a re-evaluation of our understanding. In the absence of requisite hippocampal function for simple olfactory memories, our finding of residual 'memory protein' expression, during anesthesia, in areas proposed to play a role in olfactory memory, already casts doubt on some of the underlying themes of Alkire and Guzowski's hypothesis.

In a recent review article in *Science,* Michael Alkire, Anthony Hudetz and Giulio Tononi concluded by saying: "Thus, anesthetics seem to cause unconsciousness when they block the brain's ability to integrate information (Alkire et al., 2008)." Though much depends on the definition of 'integrate' and 'unconsciousness', terms which are subject to a variety of interpretations, our data challenge the current understanding of anesthesia and memory processes and suggest that information integration and unconsciousness are not mutually exclusive.

5.3 WHY KETAMINE/XYLAZINE?

We found evidence of information integration during ketamine/xylazine anesthesia as seen by changes in c-Fos protein expression in cortical olfactory regions and changes in PSA-NCAM in the rostral migratory stream. These changes reflected those observed during the awake state. Despite these changes in protein expression, explicit memory recall was still absent in these animals. However, it is possible that an implicit or autonomic memory still persisted. So, why were we able to see these brain changes during ketamine/xylazine anesthesia and not with propofol or pentobarbital anesthesia?

Ketamine belongs to the cyclohexamine class of drugs which are categorized as sympathomimetic anesthetics (Soma, 1983). These drugs produce a cataleptic anesthetic state characterized by muscle rigidity and analgesic properties. Ketamine also produces anesthesia, amnesia, changes in autonomic function (elevated heart rate, blood pressure and respiration; bronchodilation) and tremors and seizures at higher doses; however, this is significantly minimized by the co-administration of drugs such as xylazine or diazepam (Soma, 1983; Reich and Silvay, 1989; Haas and Harper, 1992). Pharmacologically, ketamine is a non-competitive antagonist of the N-methyl-D-aspartate (NMDA) receptor, which mediates slow excitatory, glutamatergic neurotransmission (Martin and Lodge, 1985). Ketamine also inhibits the nicotinic acetylcholine receptor (nAChR); however unlike most anesthetics, with the exception of xenon and perhaps nitrous oxide, ketamine is thought to have little or no effect on GABAergic receptors (Anis et al., 1983; Krasowski and Harrison, 1999). At anesthetic doses ketamine also interacts with the μ opioid and sigma receptors, but to a lesser extent (Hirota et al., 1999; Narita et al., 2001). Xylazine is a sedative that is commonly used in combination with ketamine or other anesthetics in veterinary medicine to produce sedation, muscle relaxation and analgesia at

higher doses (Soma, 1983). Xylazine acts as an α_2-adrenergic receptor agonist which is thought to modulate norepinephrine release (Kobinger, 1978; Greene and Thurmon, 1988). The unique molecular targets of ketamine/xylazine anesthesia may help explain the effects found with this drug in our current experiments.

The exact role of specific neurotransmitter systems in altering brain circuitry and mediating the phenotypes of various anesthetics is not well understood. In the olfactory bulb, granule cells contribute to lateral inhibition through dendrodendritic and often reciprocal synapses with mitral/tufted cells, the principle neurons that mediate synaptic transmission to higher cortical regions (Mori et al., 1999; Urban and Arevian, 2009). This process is thought to facilitate the production of a coherent odor representation. Traditional GABAergic anesthetics enhance granule cell-mediated inhibition of mitral/tufted cells (Nicoll, 1972; Tsuno et al., 2008). In contrast, NMDA-antagonists, including ketamine, have been shown to reduce the granule cell mediated inhibition, thus enhancing mitral/tufted cell excitability (Wilson et al., 1996). Disinhibition of mitral/tufted cells and removal of granule cell-mediated lateral inhibition may produce a stronger, albeit incoherent, signal transmitted to the piriform cortex. This signal might then produce robust c-Fos activation that is not associated with an entirely coherent odor representation. Enhanced bulbar-piriform signaling with ketamine/xylazine anesthesia is supported by our raw c-Fos micrographs (Fig. 2-5 and 2-6) which reveal stronger (darker) c-Fos expression in individual neurons when an odorant mixture is presented during anesthesia, relative to when the odorant mixture is presented while awake. Such an interpretation may explain the elevated c-Fos immunoreactivity observed specifically with ketamine/xylazine anesthesia in the absence of an olfactory memory. Indeed, ketamine-mediated disinibition of

excitatory neurons is thought to underlie the activating effects of this drug in various brain regions (Brown et al., 2010).

Ketamine is also categorized as a dissociative anesthetic, which produces a feeling of detachment from one's surroundings. These types of anesthetics involve sensory impairment and amnesia, but can also produce unwanted side-effects. Some believe the actions of dissociatives, such as ketamine, occur without full loss of consciousness (Winters et al., 1972; Bonta, 2004). One major issue with the administration of ketamine anesthesia in humans is the prevalence of 'emergence phenomena' which includes distressing hallucinations, vivid dreams, floating sensations and delirium. This occurs in 3% to 30% of patients; however, co-administration with sedatives reduces these experiences (Reich and Silvay, 1989; Strayer and Nelson, 2008). The hallucinatory properties of subanesthetic ketamine have lead to the increased illicit use of this agent as a recreational drug (Degenhardt et al., 2005). The drug is also thought to produce schizophrenia-like symptoms and has been used as a model to study the disorder (Northoff et al., 2005; Bubenikova-Valesova et al., 2008).

Similar to other anesthetics, ketamine produces anterograde amnesia at subanesthetic doses and retrograde amnesia at higher doses (Pitsikas et al., 2008; Boultadakis and Pitsikas, 2011). Ketamine has a greater amnestic effect on males than on females (Morgan et al., 2006); however, females are more likely to experience emergence phenomena than males (Craven, 2007), a finding that may result from the differential effect of sex hormones on the NMDA receptor (Morgan et al., 2006).

As opposed to the central nervous system depression produced by most other anesthetics, ketamine produces a global activation as assessed by PET cerebral blood flow in humans (Langsjo et al., 2003; Langsjo et al., 2005), and BOLD fMRI (Littlewood et al., 2006), 2-DG and

c-Fos labeling in rodents (Duncan et al., 1998). Most anesthetics also cause an EEG state similar to deep sleep; however, ketamine produces more of an active EEG state (Schuttler et al., 1987; Maksimow et al., 2006; Tsuda et al., 2007; Franks, 2008). The active brain state associated with ketamine is thought to be produced by the inhibition of excitatory NMDA receptors on inhibitory interneurons. Ketamine specifically inhibits cortical, hippocampal and limbic interneurons, thus disinhibiting glutamatergic pyramidal neurons. This produces an uncoordinated active state thought to mediate unconsciousness (Olney et al., 1999; Seamans, 2008; Brown et al., 2010). BOLD fMRI studies in rodents revealed that administration of a nociceptive stimulus to rats fully anesthetized with ketamine anesthesia produced activation in the sensory-motor cortex, insular cortex, cingulate cortex and other regions associated with pain processing (Shih et al., 2008). An fMRI study in monkeys administered low dose ketamine demonstrated that the brains of these animals not only received visual stimuli, but were able to use the information to drive a sensorimotor loop despite behavioral dissociation (Leopold et al., 2002). Based on these studies, residual cognitive processes active during ketamine/xylazine anesthesia may allow changes in gene expression, typically occurring in the awake state, to persist without fully integrating the information within the normal cognitive hierarchy, a process that likely requires conscious perception.

The atypical molecular targets, elevated brain activity and residual processing unique to ketamine anesthesia provide several possible explanations for the c-Fos and PSA-NCAM-related information consolidation that appeared to occur during ketamine/xylazine, but not pentobarbital or propofol, anesthesia.

5.4 FUTURE DIRECTIONS

We have demonstrated changes in immediate early gene function in the anterior piriform cortex and orbitofrontal cortex that reflect olfactory experiences and persist under ketamine/xylazine anesthesia. Previous studies have demonstrated that immediate early gene expression in specific brain loci is required for certain types of memory formation (Guzowski et al., 2000; McIntyre et al., 2005). Though we have found changes with c-Fos in olfactory cortical regions that are temporally linked to memory consolidation, the functional relevance of these changes in expression is still unknown. One method to assess whether *c-fos* function is necessary for olfactory memory formation in the APC and OFC is to reduce or eliminate *c-fos* function in these regions. Focal knockdown of *c-fos* has been shown to produce greater effects on learning and memory than global gene knockout, which can lead to upregulation of compensatory genetic mechanisms (Yasoshima et al., 2006). Thus, focal knockdown of *c-fos* mRNA in the APC and OFC would be ideal to elucidate the role of *c-fos* in the particular stages of olfactory memory processing. If *c-fos* expression in one or both regions is found to be required for olfactory memory consolidation, then follow-up studies would be necessary to determine why c-Fos expression could occur in these regions under ketamine/xylazine anesthesia without leaving an explicit memory trace. Such studies could explore the function of downstream targets of c-Fos.

Much is known about the regions of the brain activated by olfaction (Savic, 2002a, b). The cortical and subcortical regions required for the storage, maintenance and retrieval of olfactory memories as well as for olfactory discrimination, however, are not well understood. The areas active during odorant stimulation with ketamine/xylazine or other anesthetics are even less well known, but may provide some insight into the regions involved in conscious perception. Future studies could address IEG function in the following regions:

- *Entorhinal Cortex* – The entorhinal cortex has efferent and afferent connectivity with the hippocampus, and connects this region to other cortical areas (Witter, 1993). Lesions to the entorhinal cortex can impair some forms of olfactory memory acqisition (Staubli et al., 1984; Staubli et al., 1986). However, others claim that the entorhinal cortex is not necessary for olfactory memory (Slotnick and Risser, 1990). Further studies found that entorhinal lesions allowed long-term olfactory memories to be made, but produced impairments in olfactory working memory (Staubli et al., 1995).
- *Perirhinal Cortex* – In the rodent, the perirhinal cortex receives considerable inputs from olfactory areas, and has connections with the orbitofrontal cortex, entorhinal cortex and hippocampus among other regions (Suzuki, 1996). This area has been shown to be required for familiarity-based recognition (Eichenbaum et al., 2007). The perirhinal cortex was also shown to produce abundant c-Fos labeling in response to an olfactory memory task despite an absence of labeling in the hippocampus (Tronel and Sara, 2002).
- *Basolateral Amygdala* – The basolateral amygdala is a limbic brain region thought to weigh incoming stimuli and memories with emotional saliency. The BLA shows a dramatic elevation in c-fos following olfactory memory acquisition (Hess et al., 1997; Tronel and Sara, 2002). Electrophysiological data shows that BLA neurons generate selective responses to odorants associated with reward (Schoenbaum et al., 1999). Additionally, the BLA has been suggested to be critical for anesthetic-induced amnesia as lesions to the BLA block propofol- (Alkire et al., 2001), sevoflurane- (Alkire and Nathan, 2005) and diazepam- (Tomaz et al., 1992) induced amnesia.
- *Locus Coeruleus* – Expression of *c-fos* shows rhythmic variations with significantly greater expression throughout the cortex during the waking (conscious) state. This

- *Hippocampus* – The hippocampus has been proposed to play a role in olfaction as this region is uniquely responsive to olfactory stimuli, over that of other modalities (Vanderwolf, 2001). Furthermore, odorant presentation can produce synchronous rhythms between the hippocampus, olfactory bulb and cortex (Macrides et al., 1982). Lesions which disrupt hippocampal function impair the formation of complex olfactory memories, but do not affect simple memory acquisition (Staubli et al., 1984; Staubli et al., 1995; Bunsey and Eichenbaum, 1996). Though olfactory associations do not cause robust c-Fos activation in this region (Tronel and Sara, 2002), it is possible that Arc may instead be activated, or that this region is involved in olfactory memory formation through IEG-independent mechanisms. Though c-Fos protein was not upregulated by non-spatial olfactory tasks, *c-fos* mRNA can be upregulated (Hess et al., 1995a, b). A similar discrepancy between mRNA and protein expression has been demonstrated in the hippocampus with the IEG, Arc (Ren et al., 2008).

- *Dorsomedial Thalamic Nucleus (DMN)* – The dorsomedial thalamic nucleus is the region of the thalamus that is involved in olfactory processing (Staubli et al., 1987a). Lesions to this region impair the ability of rats to learn new odor discriminations, however extensive training can offset this deficit (Staubli et al., 1987a; Slotnick and Risser, 1990).
- *Other Regions* – The prelimbic cortex and the habenula both have been shown to produce preferential c-Fos expression when an odor and reward are paired (Tronel and Sara, 2002), indicating a potential role in olfactory memory. The midbrain reticular formation is a region of interest as it is involved in sleep and arousal and is thought to be a target of general anesthetics (Alkire et al., 2000; Rudolph and Antkowiak, 2004). Finally, the olfactory tubercle and the anterior olfactory nucleus are also plausible candidates as these regions receive direct olfactory input from the bulb (Shepherd, 2007), however their functions are not well understood.

The NMDA receptor is also an interesting candidate for future study. This receptor is a voltage and ligand gated ionotropic glutamate receptor that has long been implicated to play an important part in learning and memory due to its role in mediating LTP (Collingridge et al., 1983). NMDA-antagonists, AP5 and MK-801, have been shown to impair memory of odor-reward, passive avoidance, Morris water maze and radial maze tasks (Morris et al., 1986; Danysz et al., 1988; Caramanos and Shapiro, 1994; Tronel and Sara, 2003). Inhibition of NMDA function was most effective when administered between 5 and 90 minutes post-training (Przybyslawski and Sara, 1997). It has been shown that an odor-reward association task produces a significant reduction in NMDA receptor density in the frontal cortex and hippocampus at 30 minutes post-training (Roullet et al., 1999). Also, several groups have shown

a relationship between NMDA receptor density and retention (Wenk et al., 1989; Keller et al., 1992; Stecher et al., 1997). While it is known that some anesthetics (*e.g.* ketamine) have direct effects on NMDA receptor function, the role of anesthesia in modulating NMDA receptor density with regard to olfactory or other memory types has not been adequately investigated.

5.5 SIGNIFICANCE

Clinically, one of the concerns in the field of anesthesiology is the rare occurrence of anesthesia awareness, which involves both consciousness during anesthesia and the ability to recall the memory (Goldmann, 1988). Indeed, there are numerous clinical reports of explicit memory formation under general anesthesia (Ghoneim and Block, 1992; Ghoneim, 2000; Sebel et al., 2004; Errando et al., 2008), especially when the information is emotionally salient (Trustman et al., 1977). Recent large-scale clinical studies have revealed an incident rate of recall following anesthesia to range from 0.1% to 1.0% (Sandin et al., 2000; Sebel et al., 2004; Errando et al., 2008; Malviya et al., 2009; Xu et al., 2009) with some estimates as high as 4% or greater (Weinberger et al., 1984). While the existence of implicit memory formation during surgical anesthesia is generally accepted (Block et al., 1988; Bailey and Jones, 1997; Kiviniemi, 1997), explicit memory formation is typically regarded as an epiphenomenon of clinical errors. Indeed, most controlled studies have not found evidence of explicit memory (Ghoneim et al., 1999; Lubke et al., 1999; Iselin-Chaves et al., 2005); however, there have been exceptions (Russell and Wang, 1997; Ghoneim et al., 2000). Those who claim to experience anesthesia awareness report pronounced anxiety during the surgical procedure. In one study, patients who experience

awareness during anesthesia were interviewed 2 years after the incident and most reported moderate to severely debilitating psychological issues that had persisted from the time of the event (Lennmarken et al., 2002). It is estimated that 26,000 new cases of anesthesia awareness occur in the United States each year (Sebel et al., 2004). Given the prevalence of this issue, the profound psychological and medicolegal repercussions, and the possibility to influence postoperative progress (Ghoneim and Block, 1992), a greater understanding of the cognitive processes during anesthesia is warranted. These reports reveal that our ability to gauge consciousness is still quite limited (Alkire et al., 2008).

Admittedly, our studies in rodents have not brought us much closer to reducing the prevalence of awareness during clinical anesthesia. However, we have continued to expand the idea of what the anesthetized brain is capable of. We have demonstrated a profound result that the molecular pathways of memory are activated despite an unconscious state. Continued study of immediate early gene function in the awake and anesthetized state may help elucidate the various brain regions necessary for anesthetic-induced unconsciousness. These data might eventually help anesthesiologists improve patient treatments and reduce anesthesia-related sequela. More importantly, however, the recent work in immediate early gene function suggests that the molecular mechanisms of consciousness are starting to be unraveled.

APPENDIX A

RECONCILIATION OF QUANTIFICATION TECHNIQUES

This appendix includes the control and extended quantifications from chapter 2. Figure A-1 demonstrates robust behavioral effects regardless of scoring technique or timeframe assessed. Automated scoring included the net time the rodent's snout spent within the 2" diameter odorant zone, independent of behavior (active smelling vs. stationary position). Manually scored behavior was performed by blinded observers looking for only active smelling behaviors within the odorant zone. The raw data from these graphs can be visualized through snout traces (Fig. A-2). Snout traces indicate the path of the rodent's snout from the first 100 seconds after approaching the odorant zone.

Figure A-3 demonstrates that data from figures 2-5 and 2-6 can be obtained with differing quantification techniques. Original stereological data (Fig. A-3A) can be replicated with a software threshold quantification technique (Fig. A-3B), or through blinded manual counting (Fig. A-3C).

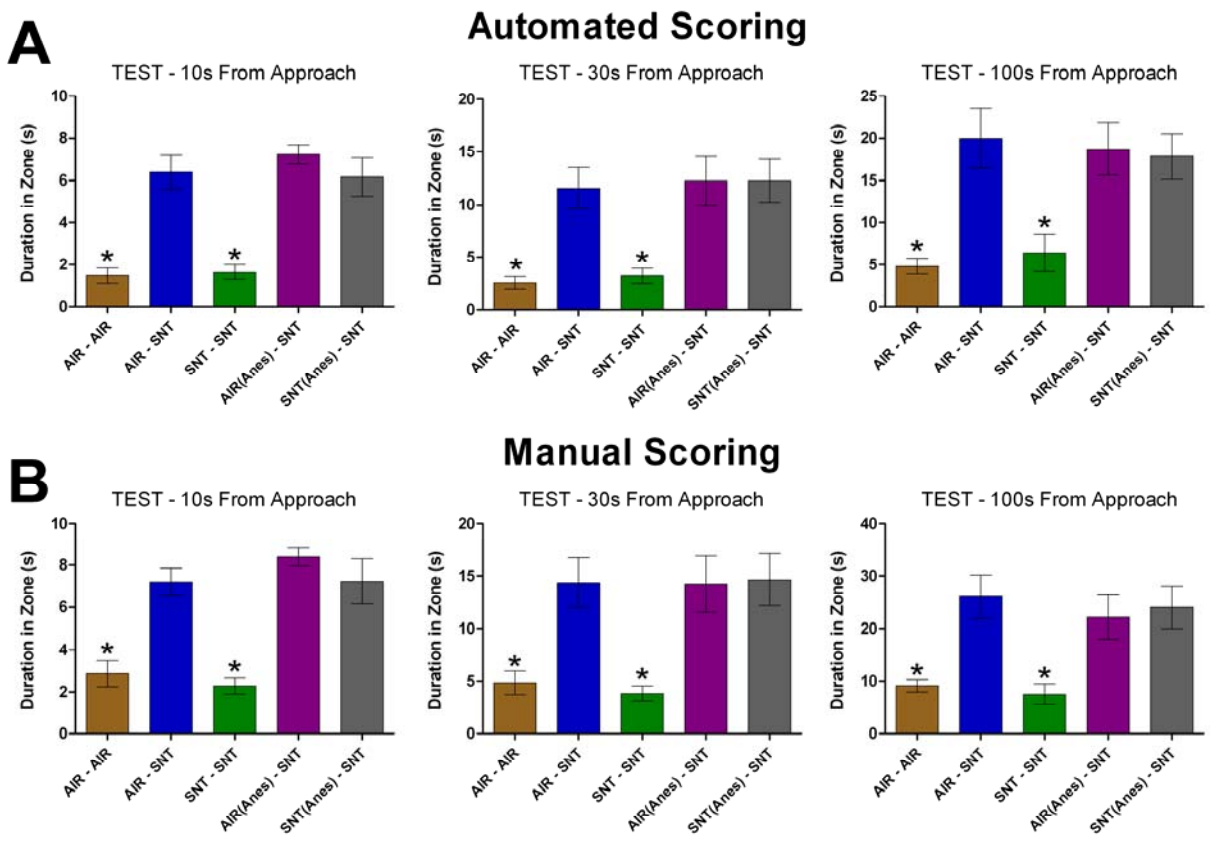

Figure A-1. Quantification of behavioral data with automated and manual scoring techniques

Odor familiarity with the odorant was assessed by duration of odor investigation time. (A) Odor investigation times as assessed by automated (A) and manual scoring (B). Smelling duration was determined by the duration of time the snout was within the odorant zone within the first 10, 30 and 100 seconds after approach. Data from experiment 'B'. (*) $p < 0.05$ relative to AIR-SNT, one-way ANOVA with Tukey *post hoc* test.

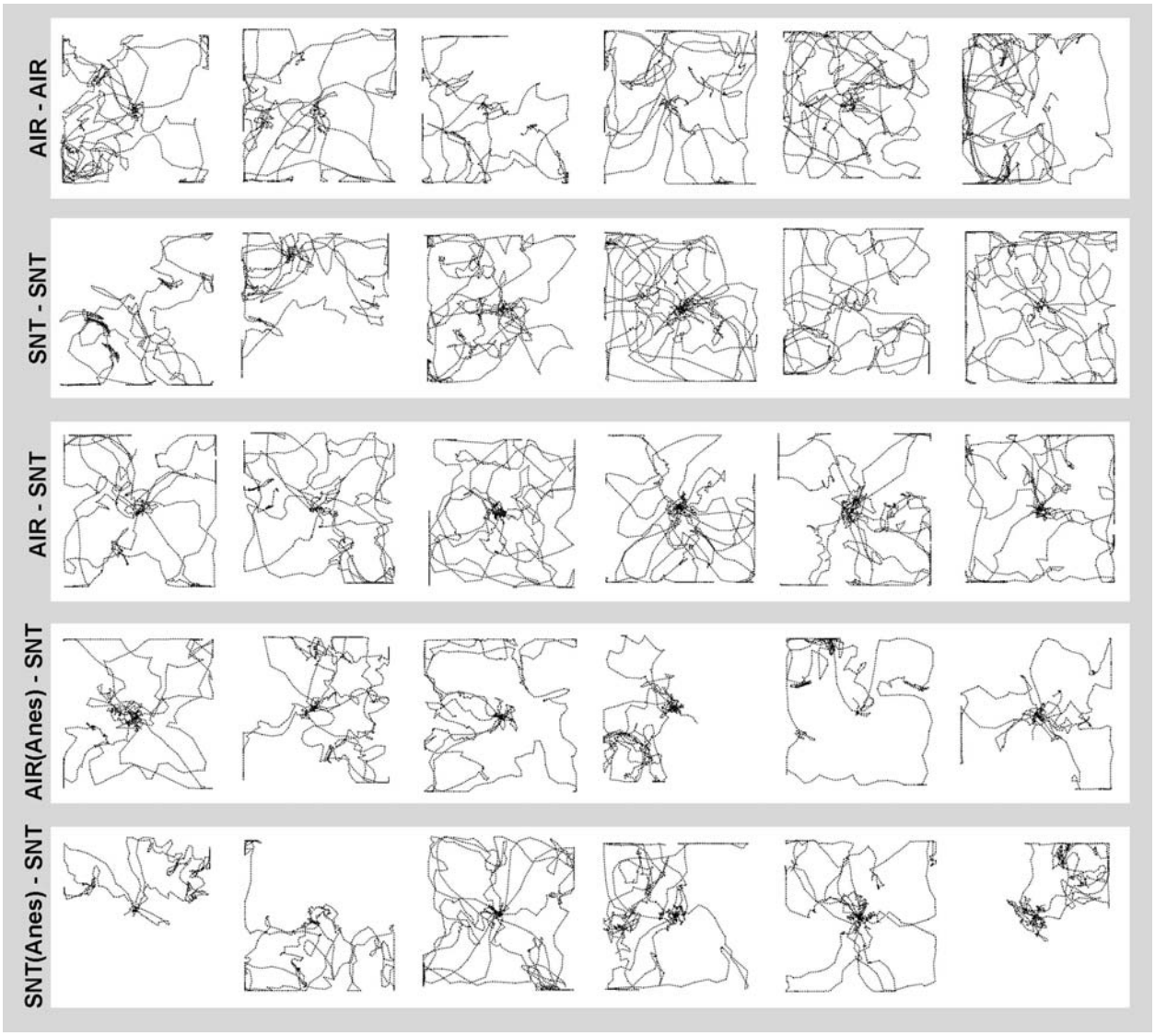

Figure A-2. Raw traces of the snout path

Path images show the position of each rodent's nose within the behavioral chamber during the test period. Traces represent the first 100 seconds following approach to the odorant zone. Data from experiment 'B'.

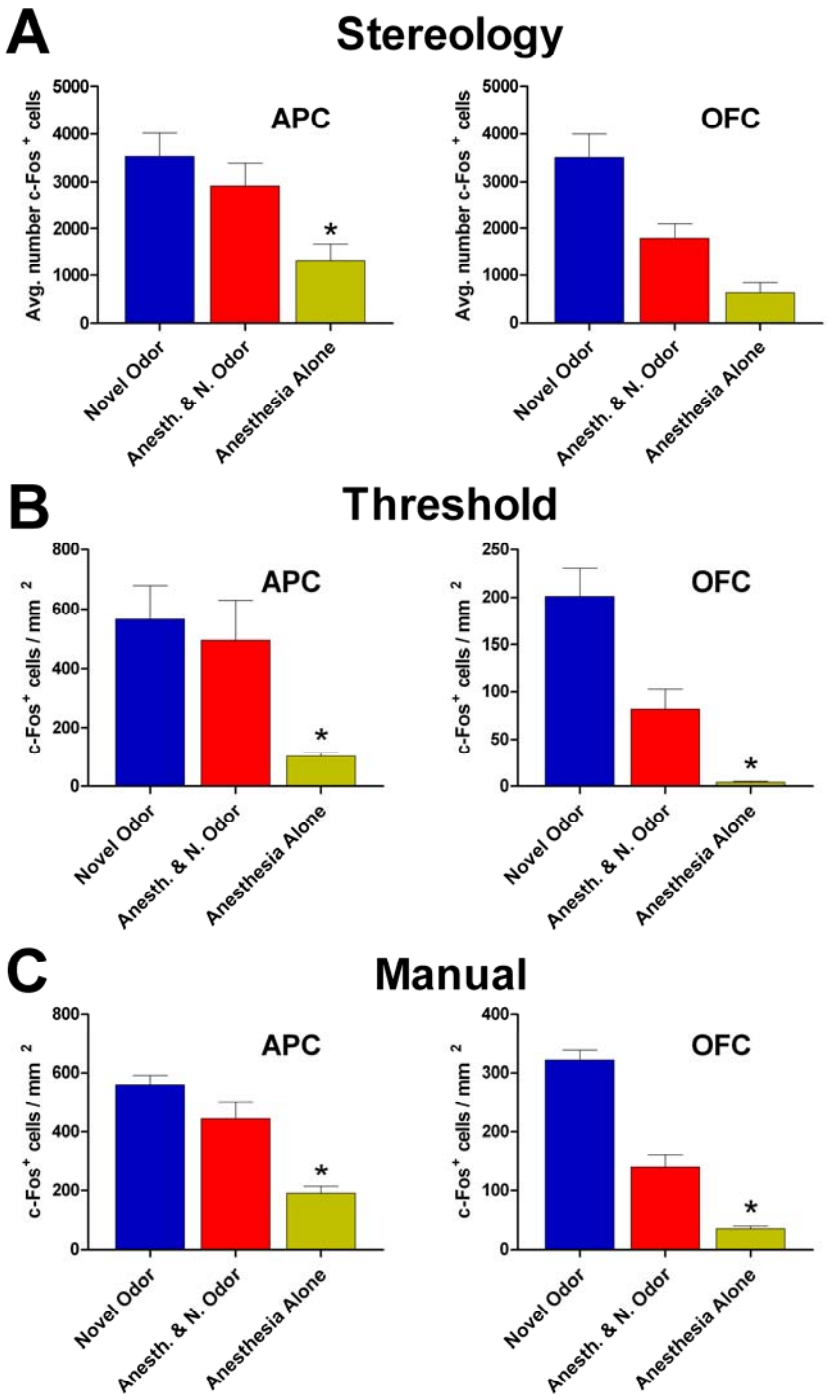

Figure A-3. Similar results of c-Fos quantification regardless of counting technique

c-Fos immunolabeling in the anterior piriform cortex (APC) and orbitofrontal cortex (OFC) as determined by (A) stereology, (B) threshold and (C) manual counting techniques. Data from experiment 'A'. (*) $p < 0.05$ relative to 'Anesth. & N. Odor', one-way ANOVA with Tukey *post hoc* test.

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