# THE IMPACT OF PRIOR EXPERIENCE WITH CROSS-MODAL STIMULATION ON ACTIVATION OF BIPOLAR AND MULTIPOLAR NEURONS IN THE INTERMEDIATE LAYER OF RAT SUPERIOR COLLICULUS

A Thesis
by
David Andrew Crane

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**Abstract**

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Multisensory integration (MI) is the process by which information from multiple sensory modalities converge on single neurons. This process allows an organism to make better use of the large amount of sensory information it receives. When a rat orients toward a cross-modal light and sound stimulus, neurons in the midbrain superior colliculus (SC) integrate the light and sound information before signaling motor planning neurons. Most cells conducting MI are multipolar, and the rat intermediate SC (intSC), which contains both bi- and multipolar neurons, plays a crucial role in MI used to guide behavior. A goal of this study was to determine the densities of bi- and multipolar neurons within intSC activated by modality specific and cross-modal stimuli. Neurons responsible for MI may not exhibit this function until experience dictates the need to do so; for instance, after an animal experiences multimodal stimuli. If cells in the intSC do not necessarily have to do MI, neural activity in intSC evoked by multimodal stimulation may be greater in animals that

have prior multisensory experience than those that do not. The second goal of this study was to investigate the impact of prior experience with multisensory stimulation on c-fos expression evoked by multisensory stimulation. This study was conducted using archival neural tissue from brains of rats exposed to modality specific (light or sound) or cross-modal (light and sound) stimulation, as well as from rats with and without prior exposure to cross-modal stimuli. The tissue was processed for c-fos, which is a protein indicating neural activity evoked by stimulation. Data collection involved counting c-fos positive bipolar and multipolar neurons in intSC, as well as counting activated cells when morphology was undetermined. More activated neurons were found in the intSC of cross-modal stimulated brains than in modality specific stimulated brains, and tissue from rats with cross-modal stimulation experience prior to stimulation to evoke activity had more activated neurons than rats that did not have prior experience with cross-modal stimulation. The results suggest that rat intSC processes information derived from cross-modal stimuli, and prior experience with sensory input from multiple modalities may influence the extent of neuronal response in the rat intSC when presented with cross-modal stimulation.

*Keywords:* multisensory integration, superior colliculus, sensory processing, evoked activity

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## Foreword

This thesis is written in accordance with the style of the *Publication Manual of the American Psychological Association (6th Edition)* as required by the Department of Psychology at Appalachian State University.

# The Impact of Prior Experience with Multimodal Stimulation on Activation of Bipolar and Multipolar Neurons in the Intermediate Layer of Rat Superior Colliculus

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## Abstract

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rats that did not have prior experience with cross-modal stimulation. The results suggest that rat intSC processes information derived from cross-modal stimuli and prior experience with sensory input from multiple modalities may influence the extent of neuronal response in the rat intSC when presented with cross-modal stimulation.

*Keywords:* multisensory integration, superior colliculus, sensory processing, evoked activity

# The Impact of Prior Experience with Cross-modal Stimulation on Activation of Bipolar and Multipolar Neurons in the Intermediate Layer of Rat Superior Colliculus

Living creatures are constantly exposed to a plethora of sensory information. An organism's ability to incorporate this information efficiently and use it for decision-making and goal-directed behavior greatly improves its chances of survival and reproduction. For example, when a rat is exploring a basement for food it must be able to avoid threats, such as an approaching cat. In this case, it is vital for the rat to incorporate information successfully from multiple sensory modalities. If the animal is able to unite multiple sensory inputs, for instance the sound of footsteps and the sight of fur, the likelihood of the animal properly acting on the external cue will change. In this case the integration of sensory inputs may help our rat to escape an approaching cat. The neural process underlying this incorporation of information is known as multisensory integration (MI). MI occurs through neural processing that combines sensory information from multiple sources and multiple sensory pathways at some single point or across a circuit (Stein & Stanford, 2008).

MI functions as part of the convergence processing that synthesizes various sensory inputs for use in perceptual, motor, and cognitive processes. Most sensory cells in the brain are unimodal and respond only to single sensory modalities such as light or sound. Some cells, however, seem to behave differently when receiving information from multiple sensory inputs, such as the combination of light and sound (e.g., Stein & Stanford, 2008). These activity changes have been coined "enhancement" and "depression" (Meredith & Stein, 1983).

The terms enhancement and depression refer to changes in the activity level of a cell as indicated by the number of action potentials over a given period of time. Enhancement

refers to the general increase in the number of action potentials measured when a second modality is presented to a cell doing MI, while depression refers to the general decrease in the number of action potentials. Meredith and Stein (1983) demonstrated that a sensory stimulus (light) presented to a subject along with a secondary stimulus (sound) will elicit enhancement or depression in the neural activation level, leading to a change in the likelihood of behavioral responses. For example, while coincident presentation of a light and sound at a target location will enhance the likelihood of an animal approaching the target accurately (Stein, Meredith, Huneycutt, & McDade, 1989), simultaneous presentation of light and sound from different spatial locations can depress activity in neurons responsive to multiple sensory inputs (Binns, Turner, & Salt, 1999), which may attenuate behavior.

The neurons responsible for MI are housed in various brain regions. Regions of the basal ganglia have been shown to mediate sensory information pertaining to motor actions (Alexander, DeLong, & Strick, 1986). The medial superior temporal area of the cerebral cortex shows evidence supporting its role in the integration of motion-based cues and goal-directed movements in the macaque (Tanaka, Fukada, & Saito, 1989). Cortical areas, such as the rostral lateral suprasylvian sulcus, often play an important role in MI leading to the top-down process of using sensory information derived from primary and association areas of specific sensory systems to direct behavior (Wilkinson, Meredith, & Stein, 1996; Zangenehpour, & Chauduhri, 2001). One of the most important regions for MI, however, is found not in the forebrain but in the midbrain tectum. The superior colliculus (SC) of many species including cat (e.g., Meredith, Nemitz, & Stein, 1987), ferret (King & Carlile, 1993), rat (e.g., Burnett, Stein, Perrault, & Wallace, 2007), and mouse (Drager & Hubel, 1975) contains neurons that exhibit the ability to do MI. The SC processes a variety of visual,

auditory, and tactile information en route to efferent motor signals directing behaviors such as gaze and orientation. Such behaviors rely on information derived from multiple sensory modalities (e.g., Burnett, Stein, Chaponis, & Wallace, 2004; Henson, 2009). However the tendency of SC neurons to respond differently to stimuli of different modalities may not be inherent. The development of the ability to process cross-modal information may rely on the development of MI neurons in the SC, which may be influenced by exposure to cross-modal stimuli in animals such as the monkey, cat, and rat (e.g., Vachon-Presseau, Martin, Lepore, & Guillemot, 2009; Wallace & Stein, 1997, 2001). The purpose of this thesis is to investigate differentially, evoked activation of neurons in rat SC by modality specific and cross-modal stimuli, and to investigate the impact of multimodal experience on evoked activity of SC neurons.

**The SC and MI**

The SC is a prominent midbrain structure that is responsible for a number of processes, many of which are MI-related. For example, the SC plays an integral role in the mediation of goal directed and orienting behavior, which can be enhanced by MI (Bell, Corneil, Meredith & Munoz, 2001; Patton, Belkacem-Boussaid, & Anastasio, 2002). Since orienting to stimuli and then using stimuli to guide behavior are critical to finding food and other resources, as well as finding or avoiding other animals, the role the SC plays is important to the basic survival functions of an organism. Proper function of the region may determine the survivability of an animal. SC function relies on a variety of sensory inputs, and the structure employs a larger than average number of sensory integration and MI neurons that contribute to the output to various brain structures (Wallace, Wilkinson, & Stein, 1996; Meredith & Stein, 1983, 1985).

The SC makes up the rostral midbrain tectum and, in rat, can be seen just inferior to the midline of the cerebral cortex (King, 2004). The SC is usually described as a layered structure that is anatomically separated into at least superficial and deep layers, which perform different primary functions. The primary role of the superficial layers, consisting of dorsal layers I-III, is integration of visual input. Neurons of these layers receive input from the retina, thalamus, and visual cortex (Patton et al., 2002; Stein, 1981) and consist of small and medium multipolar and bipolar cells (Oliver & Huerta, 1992). Multipolar neurons of the superficial layers are often described as stellate and granule (Oliver & Huerta, 1992), having a rounded appearance in Nissl-stained sections and being about the same in number as bipolar cells. In contrast, the deeper layers of SC, IV through VII, seem to contain more multipolar than bipolar neurons (Oliver & Huerta, 1992). While both bipolar and multipolar neurons make connections with areas serving various sensory modalities (Mana & Chevalier, 2001; Meredith & Stein, 1983), medium and large multipolar neurons with N-methyl-D-asparate receptors ($NMDA_{RS}$) seem to be particularly important for MI (e.g., Binns & Salt, 1996; Burnett et al., 2007). The deeper layers of the SC can be further divided into two regions, the intermediate layers and deep layers, both of which contribute to MI (e.g., Mana & Chevalier, 2001).

The deepest layers of the SC contain motor-coordination cells, which initiate movement and orienting processes, particularly saccadic eye movements, as well as some auditory processing cells (Dräger & Hubel, 1975). Patton et al. (2002) demonstrated that neurons in the deep SC were responsible for mediating sensory input and directing efferent motor responses relating to cross-modal cues. They made a distinction between unimodal and multimodal deep SC neurons in that the unimodal neurons respond to input from clearly

identified sources, or sources in which there is no ambiguity concerning its origin, while multimodal neurons serve to gather more information by responding more robustly when single modality input is not clear. These cells fire at a higher rate to inputs originating from multiple modalities (Meredith & Stein, 1983). Initial visual and auditory integration often takes place in neurons located in the more dorsal of the deep SC layers, sometimes called the intermediate SC (intSC) (Patton et al., 2002; Stein, 1981; Zangenhpour & Chaudhuri, 2001). In rat, the intSC is anatomically distinct from deeper SC, having a honeycomb appearance and receiving abundant afferents from different sensory pathways (Mana & Chevalier, 2001). The MI neurons of the SC, those in intSC in rat (Zangenehpour & Chauduhri, 2001), seem to be organized topographically in relation to specific spatial locations of their respective receptive fields and show enhanced responses for cross-modal stimuli (King & Carlisle, 1993; Meredith, 2002; Patton et al., 2002). Zangenhpour and Chaudhuri (2001) exposed rats to visual and auditory stimuli prior to immunostaining brain tissue slices for the neural activity marker zinc finger protein 268 (zif268). Higher population densities of activated intSC neurons were found in groups exposed to synchronous light and sound stimulation than where seen in groups exposed to either auditory and then visual stimulation, visual and then auditory stimulation, or no stimulation. The results indicate more prevalent visual/auditory multimodal representation across intSC neurons than any other unimodal representation. Once MI takes place the resulting neural activity no longer expresses the specific afferent information that was provided by input pathways (Meredith, 2002). Meredith discovered that bimodal neurons demonstrate differential activation patterns when stimulated by modality specific or cross-modal cues. The study noted that the activation responses for modality specific auditory stimuli were relatively weak, for visual stimuli were stronger, and for the

cross-modal visual/auditory stimuli were very strong, stronger than either unimodal presentation. This effect is known as multisensory enhancement (Meredith, 2002).

MI neurons through-out the brain demonstrate two primary response characteristics when stimulated by cross-modal stimuli as opposed to modality specific stimuli. First, enhancement refers to increased neural activation when presented with cross-modal stimulation. This change in activation level likely produces more salient efferent effects that increase the likelihood of a behavioral response; e.g., orienting towards an object (Meredith, 2002; King & Calvert 2001; Stein & Stanford, 2008). Second, depression is when a cross-modal stimulus leads to a decrease in neural activation level compared to activity with modality specific stimulation. It is thought that depression plays an inhibitory function on efferent projections, helping distinguish between individual external targets (King & Calvert, 2001; Stein & Stanford, 2008). The spatial and temporal relationships of stimuli are crucial in determining the type of response pattern observed. Stimuli of two modalities that present in relatively close geographic or temporal proximity to each other tend to produce enhancement effects; an indication that these stimuli may be from the same source. On the other hand, depression effects can be observed in conditions in which stimuli of different modalities occur at different spatial locations or different times. When there is space between activation points on a receptive field the source may be deemed as two or more independent entities. Similarly, when there is a gap in time between stimuli, the event may be interpreted as multiple external events (Driver & Spence, 2000; King & Calvert, 2001).

Enhancement and depression effects have an influence by either increasing or decreasing the likelihood of a behavior related to the stimuli occurring (Meredith & Stein, 1983). Stein and colleagues (1989) explored enhancement and depression concepts by

examining the orienting behavior of cats to a cross-modal stimulus. Their results showed that the animals were much more accurate in orienting towards cross-modal cues than to modality specific cues. But does the intSC house populations of MI neurons that function to permit or enhance behavior guided by cross-modal stimuli?

Evidence expressing the critical nature of neurons in the deeper SC layers in guiding behavioral responses to cross-modal stimuli can be found in a few studies. Goodrich, Davison, and Zrull (1999) used neurotoxin injections to destroy deeper SC cells thought to mediate auditory input. The results of the study showed that rats with the targeted neuron loss responded more poorly in sound guided navigation tasks than rats without the SC neuron loss. The authors suggested deeper SC may aid in integration of auditory input to neural pathways used to guide behavior (Goodrich et al., 1999). Burnett, Stein, Chaponis, and Wallace (2004) demonstrated that multisensory neurons in the SC are necessary for successful orienting behavior dependent upon cross-modal cues by administering ibotenate lesions targeting multipolar neurons with $NMDA_{RS}$ thought to be critical for MI (e.g., Binns & Salt, 1996). After a recovery period, cats were still able to orient to modality specific stimuli but failed to orient successfully to cross-modal cues. Henson (2009), and in earlier work with colleagues (Henson et al., 2004), showed that rats with ibotenate lesions of intSC cannot accurately locate sources of simultaneous light and sound stimuli (i.e., cross-modal) but can locate sources of light or sound cues (i.e., modality specific). Lesion studies not only provide a strong method for identifying behavioral relations with the integrity of neurons of the SC, they also allow researchers to investigate the cell-level physiology by selectively destroying particular neurons based on neurochemical features (e.g., Burnett et al., 2004; Henson, 2009). For example, ibotenic acid binds to, activates, and ultimately becomes

excitotoxic for cells containing $NMDA_{RS}$. Results of these behavioral studies can then confirm the MI role for multipolar neurons of deeper SC seen in electrophysiological studies. For example, Binns and Salt (1996) explored the ability of deep SC neurons to respond to cross-modal or modality specific stimuli under the presence of an $NMDA_R$ antagonist, D-2-amino-5-phosphonovalerate (AP5). AP5 selectively blocked $NMDA_{RS}$, while not interfering with other receptors in deep SC. The results of the study showed a 57% (SD = 5%) reduction in cell responses for cross-modal stimuli, providing strong evidence to the value of specific multipolar neurons for MI within the deep SC. The array of neurons capable of MI in adult animals are likely influenced by an animal's development.

Experience is important in guiding the development of sensory neurons (Binns, Turner, & Salt, 1999; Vachon et al., 2009). For example, MI neurons, though prevalent in the adult brain, are not present in animals until they approach adulthood (Wallace & Stein, 1997). Wallace and Stein (1997) noted that the development of these neurons takes place over time, implying that experience with cross-modal events may be important for neuronal development. While Wallace and Stein (1997) showed that neurons of cat SC only developed activity driven by cross-modal stimulation gradually, after the eyes open, earlier work demonstrated that monkeys (a species born with the eyes open) show immediate multisensory responses (Wallace and Stein, 2001). It may be that simply hearing and seeing is adequate to promote development of response enhancement in SC neurons when cross-modal stimuli are presented.

The ability of a brain region to engage in MI is particularly important for most mammals. MI enhances orienting behavior, perception, and planned action, which normally develop over time but may be disrupted by depriving exposure to sensory stimuli (e.g., Yu,

Rowland, & Stein, 2010). However, "sensory deprived" SC neurons may be able to recover MI capability with experience. Yu, Rowland, and Stein (2010) demonstrated, in young cats, that even after growing up in a developmentally-deprived environment, which was completely dark and quiet, upon exposure to cross-modal stimulation neurons responsible for MI increased in number rapidly. Their research suggests that MI neurons will develop when an animal is exposed to cross-modal stimuli, regardless of prior developmental context. Young monkeys see when born, so neural responses driven by cross-modal stimuli are present at birth (Wallace & Stein, 2001), but SC neurons in young cats only develop MI responses after the eyes open and visual input is integrated with auditory input (Wallace & Stein, 1997). Areas containing MI neurons in the ferret SC can be altered by rearing animals blind (King & Carlile, 1993). So, experiences may be necessary to initiate the expression of MI cells in the deeper SC; the intSC in rat.

**The Present Study**

The purpose of the present study was to use the neural activity marker c-fos to examine whether neurons of rat intSC show differential activation for modality specific or cross-modal stimuli, and to determine the extent to which prior multisensory experience influences the number of neurons exhibiting activity evoked by cross-modal stimulation. Neurons in the deeper SC receive inputs that permit MI and produce output that can be used to guide behavior (e.g., Burnett et al., 2004, 2007; Meredith & Stein, 1983, 1985; Wallace & Stein, 2001). In rat, the area of SC important for MI is the intSC (e.g., Mana & Chevalier, 2001; Stein, 1981), which is rich in afferents from multiple sensory pathways (Mana & Chevalier, 2001; Vachon et al., 2009; Zangenehpour & Chauduhri, 2001) and is important for behavior dependent upon MI (Goodrich et al., 1999; Henson et al., 2004). In mammals,

the MI region of SC is populated by both bipolar and multipolar neurons, which may be differentially activated by modality specific and cross-modal stimuli (e.g., Binns & Salt, 1996; Burnett et al., 2007; Zangenehpour & Chauduhri, 2001). Thus, an additional goal of the present study was to quantify numbers of both bipolar and multipolar neurons in rat intSC activated by modality specific and cross-modal stimuli. Neurons destined for MI appear to need experience with cross-modal stimulation during development to emerge better suited for engaging in MI (e.g., Yu et al., 2010). Specifically, prior experience with cross-modal stimulation may elicit more neural activation than experience limited to specific modalities and result in more MI neurons. This study used archival tissue to compare densities of neurons activated by cross and specific modality stimuli in rats with and without prior cross-modal sensory experience. There were two hypotheses:

1. A period of light, sound, or simultaneous light and sound stimulation, or a similar period in quiet and dark control conditions would evoke differing neural activity in the intSC of rats as evidenced by different bipolar and multipolar c-fos positive neuron densities.
2. Simultaneous light and sound stimulation would evoke neural activity differently in intSC of normal rats and rats with prior exposure to simultaneous light and sound stimuli as evidenced by different c-fos positive neuron densities.

## Method

### Neural Tissue

Hypotheses 1 and 2 employed archival neural tissue from two studies using Long-Evans hooded rats that was conducted in the Behavioral Neuroscience Laboratory at Appalachian State University. One set of brain sections was from a 2004 study, which was

part of a larger project that was approved by the Appalachian State University IACUC on May 16, 2002 (#01-03, M. C. Zrull, PI). The second set of brain sections were from the control groups of another study that began in 2010 and was approved by the Appalachian State University IACUC on August 4, 2010 (#11-01, M. C. Zrull, PI). The tissue was produced in 2011. Hypothesis 1 was addressed with tissue from the 2004 study, and brain sections from the 2011 study were used to address Hypothesis 2. Four sections through the intSC in each brain from each previous study were used to address the hypotheses of this research. Stimulation to evoke neural activity was identical across the 2004 and 2011 studies, and the immunohistochemical procedure used to stain activated, c-fos positve neurons was the same and used supplies from the same sources across the 2004 and 2011 studies.

**Hypothesis 1**

For Hypothesis 1, brain sections through the intSC from the 2004 study conducted to examine neural activity evoked by light (L), sound (S), and simultaneous L and S (L&S) stimulation, as well as under quiet and dark (Q&D, i.e., control) conditions, was used. In the 2004 study, both continuous and pulsed stimulation was used; Hypothesis 1 of this study was addressed using brain sections from rats exposed to pulsed stimulation. Sixteen brains from control rats and rats exposed to pulsed stimuli were available. Four brains were from control, Q&D rats, four brains were from L stimulated rats, four brains were from S stimulated rats, and four brains were from rats exposed to L&S stimulation. In the 2004 study, L stimuli were 500 ms off, 1500 ms on light flashes emitted from a 10-watt, 12V halogen bulb at 75 Lux. S stimuli were 500 ms off, 1500 ms on filtered noise (4 to 10 kHz) bursts at $65 \pm 5$ dB (A scale). The rats from the 2004 study experienced the stimuli for 120 min in a cage within

a sound-attenuating chamber in a sound-attenuating (less than 30 dB ambient), dark room (less than 1 Lux). Following stimulation the rats were euthanized using sodium pentobarbital, decapitated, and the brains were excised. The brains were cut into 50 µm sections and exposed to a standard immunohistochemical procedure to visualize neurons staining positive for c-fos, which is a neural activity marker commonly used in evoked activity studies. After non-specific binding sites were blocked, the immunohistochemical procedure employed a primary antibody to c-fos (Chemicon), a biotinylated secondary antibody (Vector Labs), a peroxidase avidin-biotin complex (Vector Labs), and finally the diaminobenzidine enzyme substrate (Vector Labs) to stain c-fos protein in neurons. The tissue sections were then dehydrated, cleared, and cover-slipped.

**Hypothesis 2**

For Hypothesis 2, brain sections through the intSC from 2011 study rats were used. Normal control rats from this study experienced 120 min of Q&D, 120 min of L&S stimulation consisting of simultaneous 500 ms off, 1500 ms L (10-watt, 12V halogen bulb at 75 Lux) and S pulses (4 to 20 kHz noise at 60 ± 2 dB), or 120 min of L&S stimulation after 20 days of exposure to cross-modal stimuli (L&S-CMX). The cross-modal exposures happened in a 1 $m^2$ open field with 25 cm walls. During exposures, rats were sometimes alone and roaming free, sometimes roaming in same-sex groups, and sometimes sitting in the home cage. The stimuli for cross-modal exposures (CMXs) consisted of simultaneous L and S bursts, which occurred in a dark (less than 1 Lux), sound-minimized room (less than 30 dB ambient). Cross-modal exposures lasted from 30 to 60 minutes. The light stimulation consisted of pulses of 75 Lux light from a 10 watt, 12V halogen bulb, and the auditory stimulation was filtered broadband noise. Four different filters were used: 4 to 12 kHz band

pass, 4 kHz high pass, 8 kHz high pass, and 0.1 to 20 kHz band pass. The L and S stimuli were pulsed. Pulse durations for each CMX session varied in a range of 10 ± 3 s on and 5 ± 3 s off. The spatial sources of the visual and auditory stimulation varied throughout the study, but were always congruent. In other words, the auditory and visual cues always emanated from the same general point in space. There were approximately 20 CMX sessions for each L&S-CMX rat.

To evoke neural activity, the rats from the 2011 study experienced Q&D or L&S (rats with and without prior CMX sessions) for 120 min in a cage within a sound-attenuating chamber in a sound-attenuating (less than 35 dB ambient), dark room (less than 1 Lux). L and S stimuli were as in the 2004 study. Following the stimulation period, rats were sacrificed using the standard euthanasia procedure (Henson et al., 2004), and the brains were cut into 50 µm sections and exposed to a standard immunohistochemical procedure to visualize the neural activity marker c-fos in neurons. As in the 2004 study, non-specific binding sites were blocked; the immunohistochemical procedure employed a primary antibody to c-fos (Millipore), a biotinylated secondary antibody (Vector Labs), a peroxidase avidin-biotin complex (Vector Labs), and finally the diaminobenzidine enzyme substrate (Vector Labs) to stain c-fos protein in neurons. The tissue sections were then dehydrated, cleared, and cover-slipped.

**Microscopy and Stereology**

To address Hypotheses 1 and 2, analysis of c-fos activated neurons within each intSC from 2004 and 2011 study brains was conducted using light microscopy and stereology procedures. In each section of each brain, six sample areas 200 µm x 200 µm x 50 µm were analyzed. As shown in Figure 1, three of these samples were in the right and three in the left

intSC. The process of identifying the proper locations within the proper brain sections from which to sample and count neurons was accomplished using two microscopes. First, intSC sections with possible sample locations were determined using a Nikon Eclipse light microscope fitted with a Pixelink 1.3 megapixel camera and a Plan Achromat 4 objective. This system was used to find appropriate sections of the intSC. Sections were selected from 30% into the SC from its caudal pole through 80% of the brain area. This represents a selection from approximately 1.9 mm to 3.2 mm rostral to interaural zero. Immunostained tissue samples were examined next to Nissl-stained, adjacent sections to allow for identification of the intSC boundaries. Confirmation of proper location was made by rostrocaudal location and the honeycomb-like appearance of the intSC (Mana & Chevalier, 2001). Four sections from each brain containing the intSC were identified for analysis. Within each section, 6 samples (200 µm x 200 µm x 50 µm each) were taken from the intSC. After the sample locations had been determined, stereology began using a higher resolution microscope and digital camera combination (Reichart-Jung Diastar with Moticam 2000, 2.0 megapixel camera) and a Plan achromat 10 objective. This microscopy system allowed for higher digital magnification (approximately 670X) of a larger area used to identify multipolar and bipolar c-fos positive cells within sample locations.

For Hypothesis 1 tissue, C-fos positive, bipolar and multipolar neurons were identified by their morphology and by size. If a neuron had two processes projecting from opposite poles of the soma, it was considered a bipolar cell, and if the cell had evidence of more than two processes it was counted as a multipolar cell, as shown in Figure 2. For bipolar cells, cell body shapes that constituted a countable neuron included the typical rounded shape with two clear poles and a more fusiform shape (cylindrical or fuselage-like).

Multipolar morphologies were more diverse. Countable shapes included round, multi-angular or polygonal, and fusiform; however, in each case the cell body had three or more processes. The second factor that was used to identify a countable neuron involved its size. Very small cell fragments or other small stained bodies were excluded from the counting procedure. To assess Hypothesis 1, any stained body counted as a c-fos positive neuron needed to be large enough to have a clearly identifiable morphology.

For Hypothesis 2, immunological staining of available tissue failed to elucidate neuron morphology. Rather, staining of c-fos protein was evident within the nuclei of neural cells. Thus, a critical factor used to identify a countable neuron involved its size. Similar to procedures for Hypothesis 1 tissue, very small cell fragments or other small stained bodies were excluded from counting. This was accomplished by not counting very small "stained" objects. To correct neuron densities for non-neuronal objects erroneously counted as neurons, a correcting subtraction factor was determined for each brain. This value was found by sampling the medial lemniscus (ML) of each brain, which should contain mainly axons and in theory no neural cell bodies (i.e., no nuceli), as a reference point. The ML fos-positive density value was subtracted from each sample count obtained within each section of each brain from the 2011 study tissue to correct for possibly counting objects that were not neurons as neurons. Because Hypothesis 2 tissue contained only nuclear staining of c-fos, the correction factor helped assure that density counts were as accurate as possible.

The counting of c-fos positive cells within each sample followed strict stereological procedures. For each sample, an inclusion frame (see Figure 2), was placed over the digital image of an area of the brain section containing areas to be sampled. Using the inclusion frame to create samples prevented neurons from being counted multiple times. This

inclusion frame was systematically moved through the left and then right intSC of each brain section to create samples during the cell counting process. Material that was considered inside the inclusion frame was required to lie within the acceptance box (see Figure 2) without touching any rejection lines (the longer lines in Figure 2). Figure 2 demonstrates how neurons would be counted (green checks in the figure) and would not be counted (red checks in the figure) in the density calculated for a particular sample. The size of the counting frame (200µm x 200µm x 50µm each) was smaller than the entire field of view in any digital image of an intSC section in order to hedge against any edge effects. Any cells in the initial plane of focus (the reference plane) were not counted, and the reference plane was used to designate a set starting point from which to begin counting c-fos positive neurons. Only cells in the deeper focal planes were counted. The top of the soma was the indicator of that cell's plane of focus. In sum, a neuron was only counted if it was positive for the c-fos protein, was a bipolar or multipolar neuron, and was large enough to be identified as such, lay within the inclusion area, and lay in a focal counting plane. At most only two focal planes were observable in any given sample.

## Results

The data obtained from the 2004 study and used to address Hypothesis 1 fit into a hierarchical design with brain section nested within brain, which was nested within stimulation group. There were six density observations per section from four sections of each brain resulting in 24 density observations from each brain. All available brains with identifiable c-fos stained neurons yielded 24 observations from intSC. Two dependent variables, activated neuron density (the combined c-fos positive multipolar and bipolar neuron densities) and proportion of activated multipolar neurons (c-fos positive multipolar

cells relative to all stained neurons), were compared across L, S, L&S and Q&D stimulation conditions to evaluate Hypothesis 1.

The data obtained from the 2011 study and used to test Hypothesis 2 also fit into a hierarchical design with brain section nested within brain and brain nested within stimulation group. While most brains yielded 24 density observations (six observations per section from four sections of each brain), the staining of some sections was such that accurate identification of neurons with c-fos positive nuclei became impossible and densities from those sections were not used in analyses. One L&S section, two sections from L&S-CMX, and four sections from Q&D brains were excluded from the study. Hypothesis 2 was tested by comparing a single dependent variable, c-fos positive neuron density, across L&S, L&S-CMX, and Q&D groups. For analyses of both Hypothesis 1 and 2, ANOVA was used to compare the specific dependent measures across stimulation groups and identify differences among the groups. Significant main effects of stimulation group on general neuron density were followed by post hoc comparisons to explore specific differences. Type I error rates were set so that $\alpha = .05$ across the family of tests for the stimulation group effect on each dependent variable.

Hypothesis 1 stated that a period of light, sound, simultaneous light and sound stimulation, or a similar period in quiet and dark control conditions would evoke differing neural activity in the intSC of rats as evidenced by different bipolar and multipolar c-fos positive neuron densities. Figure 3 shows sample neuron density plots for the L, S, L&S, and Q&D conditions. A test of the hypothesis revealed a difference in activated neuron densities among the stimulation groups, $F(3,52) = 3.99$, $p < .0125$. A post-hoc contrast demonstrated more activated neurons among the stimulation groups (L, S, L&S) than in the non-stimulated

condition (Q&D), $F(1,52)=9.43$, $p < .0034$. The contrast accounted for 78% of the neuron density variance among the stimulation groups. Table 1 shows the mean neuron densities for L, S, L&S, and Q&D conditions.

Hypothesis 1 implied that proportions of c-fos positive (i.e., activated) bipolar and multipolar neurons would differ based upon stimulation condition. This hypothesis was tested by examining differences in the proportion of multipolar neurons between the groups. There was a significant difference, $F(3,52) = 4.32$, $p < .0086$, in the proportion of multipolar to other neurons between the stimulation groups (see Table 2). A post-hoc contrast demonstrated a higher proportion of multipolar neurons to other neurons in the L&S and Q&D groups than in the L and S groups, $F(1,52) = 10.32$, $p < .0023$, which accounted for 95% of the variance explained by the stimulation effect. In particular, 22% more multipolar neurons were activated in the lateral aspects of the intSC by cross-modal L&S stimulation than by single modal L or S stimulation.

Hypothesis 2 stated that simultaneous light and sound stimulation would evoke neural activity differently in the intSC of normal rats and rats with prior exposure to simultaneous light and sound stimuli as evidenced by different c-fos positive neuron densities. Figure 4 shows example neuron density plots for the L&S, L&S-CMX, and Q&D conditions. Differences in activated neuron counts between L&S, L&S-CMX, and Q&D groups were observed, $F(2,46) = 8.59$, $p < .0007$ (see Table 3). Post-hoc pairwise comparisons demonstrated a difference in c-fos positive neuron densities between the L&S and L&S-CMX groups ($t(48) = 3.85$, $p < .0004$) and between the Q&D and L&S-CMX groups ($t(48) = 2.86$, $p < .0063$). There was no statistically significant difference in activated neuron counts between the L&S and Q&D groups ($t(48)$, $p > .1096$). Overall, experience with cross-modal

stimulation before experiencing the L&S stimulation condition (L&S-CMX rats) increased activated neuron density by 21% over stimulation without prior experience (i.e., the L&S condition, see Table 3).

## Discussion

The results of testing Hypothesis 1 demonstrated that animals exposed to stimulation showed greater c-fos expression than animals exposed to no stimulation. This result suggests the intSC does process visual and auditory information above baseline (no stimulation) levels. The finding supports previous research and reinforces the role of the intSC as part of sensory processing pathways in the brain (Dräger & Hubel 1975; Meredith & Stein, 1986; Wallace & Stein, 1997; Yu, Rowland & Stein 2010).

Differences between the proportions of multipolar neurons, which depended upon the nature of the stimulation, were observed. Post hoc analyses revealed differences between the modality specific groups and the cross-modal groups, which accounted for 95% of the variance in observed densities of activated neurons suggesting that multipolar cells were more active in the cross-modal than modality specific stimulation groups. The significantly higher proportion of multipolar cells activated in the cross-modal groups compared to the modality specific groups supports the necessity of multipolar cells in the processing of information from multiple inputs (Meredith & Stein, 1986) and supports the hypothesis that multipolar cells are activated in higher proportions than other cells when cross-modally stimulated. There was no difference in activated multipolar neurons between the Q&D and L&S groups, indicating the Q&D group may demonstrate a baseline level of active multipolar cells across all groups due to the presence of another source of uncontrolled sensory stimulation such as somatosensory input (Chalupa & Rhoades, 1977; Meredith &

Stein, 1986, Dräger & Hubel, 1975; Finlay, Schneps, Wilson, & Schneider, 1978; Crish, Comer, Marasco, &Catania, 2003).

Hypothesis 2 tests showed differences between the L&S and L&S-CMX groups as well as between the L&S-CMX and Q&D groups. These differences suggest that prior experience with multimodal stimulation influences neuronal activation (Wallace & Stein, 1997; Yu et al., 2010). Specifically, that without prior experience, intSC neurons are not differentially activated by light and sound stimulation over and above no stimulation. It may be that until the visual and auditory pathways innervating the intSC are exposed to light or sound, c-fos in neurons is not expressed in greater densities than when exposed to no stimulation.

Interestingly, this study observed effects of prior experience in adult animals (rats > 90 days post natal). In Yu et al., (2010) animals (cats) were reared in darkness until 7 to 12 months of age before they were exposed to multisensory stimulation. They observed multisensory enhancement effects within hours of multisensory stimulation. Their work demonstrated that neurons, in animals sensory deprived from birth, rapidly develop the ability to do MI when exposed to multisensory stimulation. The results of the present study add to their finding by demonstrating changes in rats that are fully developed when they receive the type of sensory experience necessary to elicit multimodal responses. This research suggests that fully developed animals can also demonstrate an effect of experience on the multimodal response characteristics in superior colliculus cells. Furthermore, this study suggests that sensory deprivation may not be necessary to observe such experience dependent changes in activation characteristics.

Together, the results support the hypotheses of the study. A period of light, sound, or simultaneous light and sound stimulation did evoke neural activity in the intSC of rats more than a similar period in the quiet and dark, as evidenced by different multipolar c-fos positive neuron densities. Additionally, more multipolar neurons were active in the cross-modal than modality specific stimulation groups. Lastly, simultaneous light and sound stimulation did evoke neural activity differently in intSC of normal rats compared to rats with prior exposure to simultaneous light and sound stimuli. Animals with prior experience with cross-modal stimulation exhibited more activated cells than animals with no prior experience with cross-modal stimulation.

Results of the study also revealed interesting lines of research that should be explored further. For example, no differences in the proportion of activated multipolar neurons between L&S and Q&D groups were observed in hypothesis 2. Brains used in this study came from animals free to roam in the stimulation cage and may have been receiving uncontrolled sensory information. Perhaps the region of interest in the lateral aspect of the intSC houses cells responsible for processing other sources of sensory information aside from just visual and auditory, such as somatosensory. Future research should explore the role of prior experience with somatosensory stimulation on activation of c-fos in the intSC. These observations bring up a few points related to the topographic consistency of the region, the stimulation parameters that may influence cellular activity, the efficacy of c-fos as a target for morphological staining in MI research, and the difficulty with differentiating sources of activation.

Perhaps function of the intSC is not topographically consistent. Most research conducted on MI employs electrophysiological techniques targeting individual cells and

assessing them for their MI capabilities (Meredith & Stein, 1983; Meredith & Stein, 1986; Xu, Yu, Rowland, Stanford, & Stein, 2012; Yu, Rowland, & Stein, 2010). Perhaps the distribution of cells conducting MI is variable, with some cells in each SC sub region processing MI differentially based on sensory input. It may not only be that the SC processes sensory information selectively in different regions, but the cells that do MI in the area are not consistently distributed. For example, it has been stated that different areas of the SC respond to different spatial locations (King & Carlisle, 1993; Meredith, 2002; Patton et al., 2002; Comoli, Das Neves Favaro, Vautrelle, Leriche, Overton, Redgrave, 2012). Therefore, the stimulation source location may play a large role in how the SC as a whole expresses activation. Comoli et al. (2012) suggest that stimulation in the upper visual field elicits activity in the medial SC while stimulation in the lower visual field elicits activity in the lateral SC. In this study, the stimulation source was not a directly identifiable, acute point in space (broadband noise and bright light), and was presented from the upper visual field. This type of stimulation would, according to Comoli et al. (2012), most likely activate regions in the medial SC more readily than in the lateral SC. By focusing on the lateral aspects of the intSC, the areas impacted by the prior experience model used to produce tissue, may have overlooked activated neurons in the medial SC. Follow up research should test this potential.

The topography of the SC may also allow for extraneous information to elicit expression of c-fos within the region. For example, Chalupa and Rhoades (1977) suggest that the more caudal regions of the deep SC respond more readily to auditory input than more rostral regions. The region of interest in this study (lateral aspect of intSC) varied caudally from the initial section as much as 1.2mm. Such variety may target the suggested areas of auditory input but may also miss them in the more rostral sections. The SC has also been

shown to process visual, auditory, somatosensory (Wallace & Stein, 1997), proprioceptive (Yan, Okito, Yamaguchi, 2010), and nociceptive (Redgrave, Telford, Wang, McHaffie, & Stein,1996) information; all of which may have been sources of sensory input subsequently initiating c-fos expression in our model. Future research should experimentally manipulate multiple sources of input in an effort to further understand topographical patterns related to sensory processing in the region.

The points of topographic consistency and the levels of extraneous activation lead to a follow-up question concerning the types of stimulation necessary to elicit activation in the lateral SC. Though most of these sources of information were controlled in this study, they may all have been processed differentially during the testing stimulation period. For example, as one animal may have directly faced the light source another may have turned its back towards it, altering the way in which the stimulus was processed. Also, since the SC processes information en route to motor output, it is likely that only the most salient sensory information activates cells in the SC, which at times may not have been the intended light and sound stimulation (Stein, 1981). In this study cross-modal experience was given using a large light source, considering the space in which the rat roamed, and an acoustic source that was non-localized. Hence, an animal did not have a specific point in space as a target for the light or sound source. Furthermore, since the SC is topographically organized, the entire structure should be activated by such global stimulation (King & Carlisle, 1993; Meredith, 2002; Patton et al., 2002). It is possible that the source location of the stimulation may alter activation densities within the SC. Future research should explore the types of prior experience necessary to elicit differential activation of cells in the SC by specifically

examining the role of stimulation source location and how that might interact with the type of sensory input.

Another point that should be considered is the use of the immediate early gene (IEG) c-fos as a target for IHC staining. Zangehneopur and Chaudhuri (2002) describe the time course of c-fos expression (and related IEG zif268) as indicated by the expression of stained mRNA and protein. Their research supports our use of stimulation durations of 120 minutes prior to sacrifice. However, it would be interesting to see if there are differences when using another IEG such as zif-268. Also, some research has identified differences in the cellular distribution of c-fos. For example, Tian and Bishop (2002) identified dendritic and somatic cytoplasm staining in Purkinje cells, but not in other neuronal or glial cells in the rat cerebellum, suggesting cell-type differences in c-fos production. Chaudhuri (1997) has noted a number of variables influencing IEGs in activation assays including expression specificity, temporal activation patterns, and the stimulus coupling uncertainty, all of which would be interesting focal points for future research.

## Conclusion

An analysis of neuronal activation in the intSC supported the role of multipolar cells as major MI processing units and suggested that prior experience may be necessary for neurons of the intSC to differentially process cross-modal stimulation or modality specific stimulation. In the intSC, stimulation with light and sound elicited more cellular activation than exposure to quiet and dark, and prior experience with cross-modal stimulation elicited higher proportions of activated neurons compared to no prior experience. Further research is needed to identify differential impacts of prior experience with other sources of sensory input

(i.e., somatosensory or nociceptive input) and stimulation characteristics (i.e., spatial location, amplitude, intensity, etc.) on activation densities in the intSC.

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Table 1

*C-Fos Neuron Densities from the Lateral Intermediate Gray Layer of the Superior Colliculus for Stimulation Groups from Hypothesis 1*

| Stimulation Group | $N_{Rats}$ | $N_{Observations}$ | *M* | *SD* |
|---|---|---|---|---|
| Light Only | 4 | 96 | 14.02 | 5.58 |
| Sound Only | 4 | 96 | 15.86 | 5.04 |
| Light & Sound | 4 | 96 | 15.68 | 5.51 |
| Quiet & Dark | 2 | 48 | 10.94 | 4.97 |

Table 2

*Proportion of C-Fos Positive Multipolar Neurons from the Lateral Intermediate Gray Layer of the Superior Colliculus for Stimulation Groups from Hypothesis 1*

| Stimulation Group | $N_{Rats}$ | $N_{Observations}$ | *M* | *SD* |
|---|---|---|---|---|
| Light Only | 4 | 96 | 0.32 | 0.20 |
| Sound Only | 4 | 96 | 0.27 | 0.14 |
| Light & Sound | 4 | 96 | 0.36 | 0.16 |
| Quiet & Dark | 2 | 48 | 0.36 | 0.22 |

*Note*: The proportion of multipolar neurons is of all identifiable neurons within the lateral intermediate gray layer of the superior colliculus.

Table 3

*C-Fos Neuron Densities from the Lateral Intermediate Gray Layer of the Superior Colliculus for Stimulation Groups from Hypothesis 2*

| Stimulation Group | $N_{Rats}$ | $N_{Observations}$ | *M* | *SD* |
|---|---|---|---|---|
| Light & Sound | 4 | 90 | 17.83 | 4.23 |
| Light & Sound after Cross-modal Exp. | 6 | 132 | 21.52 | 5.12 |
| Quiet & Dark | 4 | 72 | 16.21 | 5.71 |

*Note*: Rats in the Cross-modal Exp. and Light & Sound group were those animals exposed to cross-modal (i.e., light and sound) stimulation during 20 sessions (see Method for description) prior to the final stimulation session with light and sound.

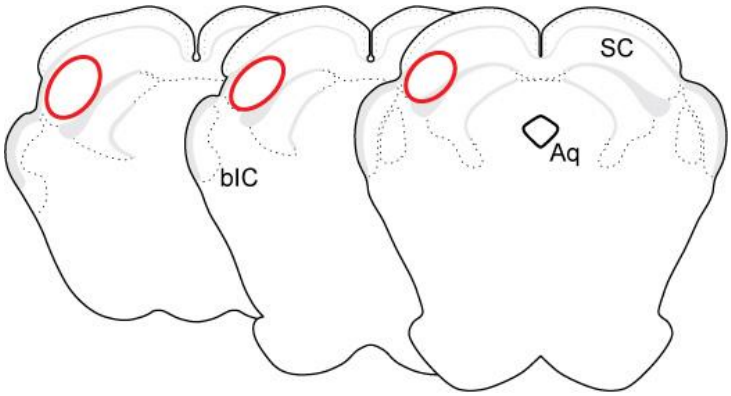


*Figure 1.* Sampling area. This figure shows drawings of serial sections through the SC of rat. The red circles indicate the area in the intermediate SC that was sampled for c-fos positive neurons. To avoid confusion sample areas are shown on only one side of the brain; however, samples were taken bilaterally. Abbreviations: Aq, cerebral aqueduct; bIC, brachium of the inferior colliculus; SC, superior colliculus.

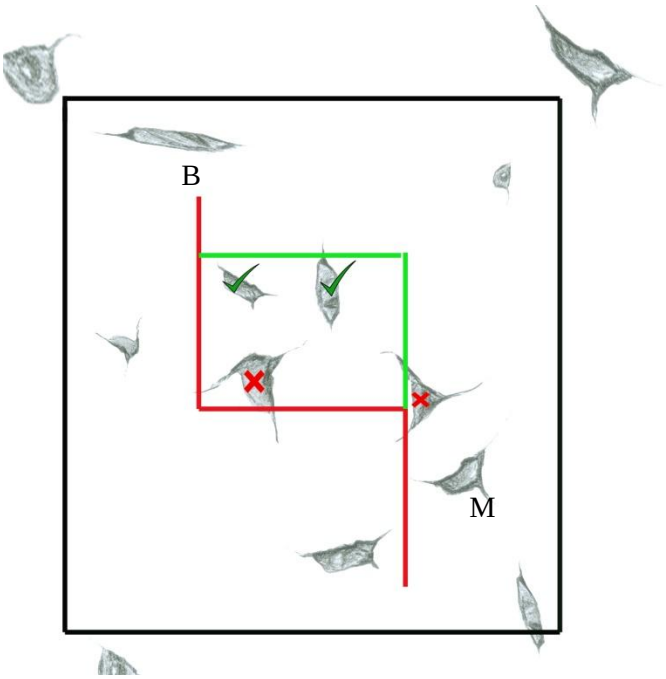


*Figure 2.* Counting frame. This figure shows a sample counting frame used in the quantification of c-fos positive bipolar (B in figure) and multipolar (M in figure) neurons. Red lines indicate exclusion areas and the green lines indicate inclusion areas. In order for a cell to have been counted it must have fallen, at least partially, inside the counting frame without touching or lying outside the red lines.

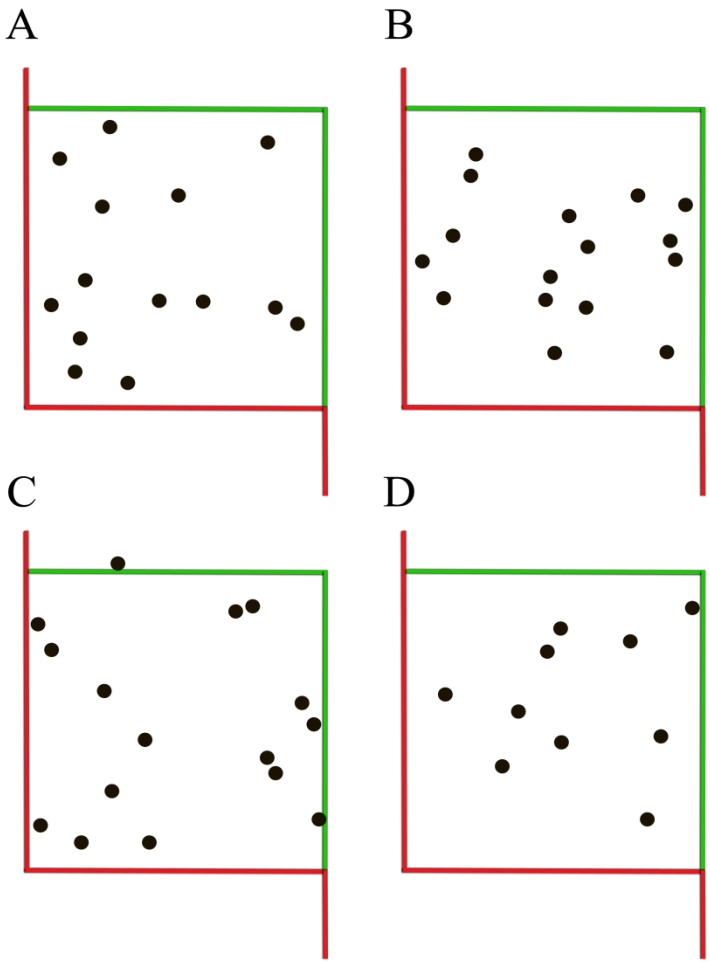


*Figure 3*. Hypothesis 1 representative dot plot. Dot plot showing representative samples from the Hypothesis 1 data. A) Rat 0412 from the light only group (L in the text). B) Rat 0410 from the sound only group (S in the text). C) Rat 0414 from the light and sound group (L&S in the text). D) Rat 0408 from the quiet and dark group (Q&D in the text).

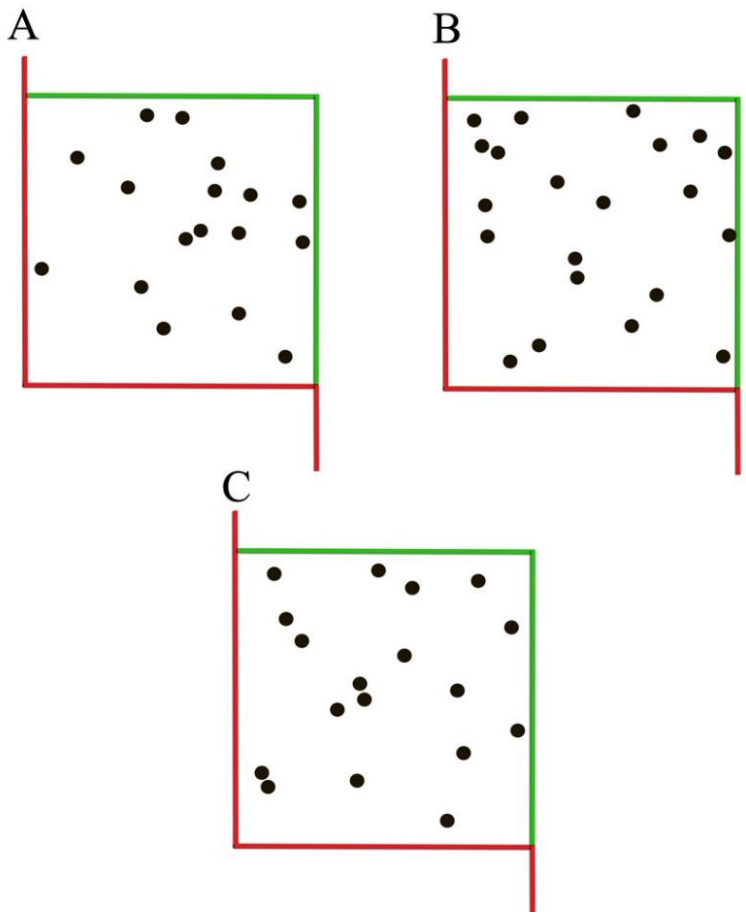


*Figure 4.* Hypothesis 2 representative dot plot. Dot plot showing representative samples from the Hypothesis 2 data. A) Rat 1104 from the light and sound stimulated group (L&S in the text). B) Rat 1111 from the group with prior cross-modal experience (CMxLS in the text). C) Rat 1103 from the quiet and dark group (Q&D in the text).

# Vita

David Crane was born in Durham, NC, in 1988. He graduated from South Point High School in Belmont, NC, in 2006 subsequently entering Appalachian State University to study Psychology and Marketing. In 2010 he was awarded a Bachelor of Science Degree in Psychology. The following fall he began studying for a Master of Arts degree in General Experimental Psychology at Appalachian State University. The M.A. degree was awarded in August 2013.