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Reema Panjwani March 30, 2016

Psychomotor activation levels in mice are regulated by vasoactive intestinal peptide produced by bone marrow derived blood cells

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Abstract

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Produced by both neurons and lymphocytes, vasoactive intestinal peptide (VIP) is a 28-amino acid neuropeptide that decreases Th1 and anti-viral immunity. VIP also plays a role in regulating neurological behavior; previous investigations have determined that VIP/peptide histidine isoleucine knockout (VIP-KO) mice exhibit altered circadian rhythms, decreased mobility, and selective cognitive disabilities. The goal of this study was to understand the role of VIP in murine behavior and determine whether VIP produced by bone marrow-derived blood cells influence neurological function. This question was addressed using radiation chimeras of female C57BL/6J VIP-KO and wild-type (WT) mice receiving syngeneic transplants of bone marrow derived cells from either VIP-KO or WT donors. We hypothesized that the behaviors of VIP-KO mice will be changed to resemble wild-type mice in radiation chimeras engrafted with hematopoietic cells from WT mice and the behavior of wild type mice will be changed to resemble VIP-KO mice in radiation chimeras of WT mice engrafted with hematopoietic cells from VIP KO mice. Mice were observed for circadian rhythms, examined during forced swim tests (FST), and tested in contextual fear conditioning (CFC) paradigms 10-weeks post-transplant. Our hypothesis was supported and a difference was noted between transplant groups receiving WT and KO hematopoietic cells. Chimeras receiving KO bone marrow-derived cells exhibited significantly greater novelty induced locomotion. Wild type chimeras engrafted with KO cells showed significantly greater dark phase activity and swam significantly more during the FST. No difference was measured in the freeze response among radiation chimeras during the CFC. These findings indicate bone marrow-derived blood cells influence behavior and that VIP produced by blood cells is important in psychomotor activation levels. Future investigations include repeating experiments with male models and analyzing VIP content and brain histology to determine neurological function during behavioral testing.

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INTRODUCTION

Brain behavior is regulated through a variety of hormones, neurotransmitters, and neuropeptides. Common neurotransmitters that help to communicate messages across nerve cells include glutamate, dopamine, acetylcholine (ACTH), norepinephrine, serotonin, and gamma aminobutryic acid (GABA). In investigations of murine cognitive behavior, these neurotransmitters exhibited an impact on learning and memory (Myhrer, 2003). Similarly, neuropeptides such as melanocortin, corticotropin-releasing factor, and glucagon-like peptide 1 all influence feeding behavior in humans (Inui, 1999). Although these particular neuropeptides were released by neurons to influence signaling, other peptides released by both central nervous system (CNS) and peripheral nervous system (PNS) neurons regulate behavior in a non-signaling manner. Production of such peptides is regulated by both the neuroendocrine and immune systems.

Although peptides and receptors are produced by both systems of the bidirectional network, the function of neuroimmune mediators is yet to be fully explored. The bidirectional network between the neuroendocrine and immune systems allows for common peptides and receptors of the two systems to act as neuroimmune mediators. Some neuroendocrine peptide hormones are understood to be endogenously produced by the immune system (Weigent & Blalock, 1987). This relationship was first indicated by the production of ACTH and endorphins in macrophages and lymphocytes, originally thought to be produced by only neurons and endocrine cells (Smith & Blalock, 1988). The demonstration of a mutual biochemical language between the two systems has made it difficult to determine distinct boundaries for each system (Delgado, Pozo, & Ganea, 2004).

Immune cells produce over 30 neuroendocrine mediators including growth hormone, prolactin, proenkephalin A, oxytocin-vasopressin, atrial natriuretic peptide, pituitary adenylate cyclase-activating polypeptide (PACAP), and of relevance to this study, vasoactive intestinal peptide (VIP)(Abad et al., 2002; Cutz, Chan, Track, Goth, & Said, 1978; Geenen et al., 1987; Montgomery et al., 1987; Rosen, Behar, Abramsky, & Ovadia, 1989; Vollmar, Colbatzky, & Schulz, 1992; Weigent & Blalock, 1987). VIP is a 28-amino acid neuropeptide, endogenously produced by the CNS, PNS, and cells of the immune system with a half-life of 3 minutes (Cutz et al., 1978; Giachetti, Rosenberg, & Said, 1976; S. I. Said & Mutt, 1970). VIP and PACAP are both members of a superfamily of structurally related peptide hormones and bind to three G-protein coupled receptors, PAC_1, $VPAC_1$, and $VPAC_2$ (Harmar et al., 2012; Langer & Robberecht, 2007).

VIP's Function in the Immune system and Presence in the CNS

VIP was originally isolated by Sami I. Said in 1968 as a vasodilator peptide from lung extracts of patients suffering from severe lung injury or massive pulmonary embolisms (Piper, Said, & Vane, 1970). Because both the upper intestine and the lungs originate from the same embryonic bud, Said recruited the efforts of Viktor Mutt and the two scientists extracted the vasodilator from the duodenum, subsequently giving the peptide its name (S. I. Said & Mutt, 1970). Soon after, Said and Rosenberg identified the production of VIP in the central and peripheral nervous systems, categorizing VIP as a neuropeptide functioning as a neurotransmitter and neuromodulator (Sami I Said & Rosenberg, 1976). Decades of subsequent research has established VIP's action in regulating systemic vasodilation, increased cardiac output, bronchodilation, hyperglycemia, smooth muscle relaxation,

neurotropic effects, learning and behavior (as will be subsequently described), bone metabolism, and some differential effects on secretory processes in the gastrointestinal tract and gastric motility (Delgado et al., 2004). Particularly, VIP and PACAP exhibit anti-inflammatory properties (Waschek, 2013). Previous work by our group indicates the increase of VIP dosage decreasing T cell proliferation and thereby decreasing inflammation and limiting anti-viral immunity (J. Li et al., 2015). We have has also published that the absence of VIP in hematopoietic cells enhances murine Th1 polarization and antiviral immunity (J. M. Li et al., 2011). Using VIP knockout (VIP-KO) mice and radiation chimeras engrafted with syngeneic VIP-KO hematopoietic stem cells (HSTC), Li et al. found that wild-type (WT) mice engrafted with VIP-KO HSTCs exhibited greater T cell immune responses in response to cytomegalovirus (CMV) infection compared to control WT→WT transplant recipients. These data suggest that VIP produced by neuronal cells does not suppress adaptive immune response in the periphery. Inflammation in the lymphoid microenvironment augments the production of VIP, making it worth investigating whether inflammation and stress might lead to VIP regulation in the CNS (Martinez et al., 1999). While VIP-KO hematopoietic cells increase adaptive immunity in bone marrow transplant murine models, VIP is produced by CNS neurons to influence neurological behaviors.

In a qualitative study of the distribution of VIP receptors in the murine (in addition to other vertebrates) CNS, researchers discovered that there was a high density of binding sites in the olfactory bulb, external layers of the cerebral cortex, dentate gyrus, midline thalamic nuclei, geniculate nuclei, some hypothalamic nuclei, superior colliculus and locus coeruleus. Intermediate densities of receptors were found in the amygdala, caudate-putamen, septum, nucleus accumbens, CA1-CA3 fields of the hippocampus and central gray.

High densities of VIP binding sites were found in the cerebellum. These findings suggest the significance and importance of VIP in the processing of specific sensory inputs by the outlined brain regions (Dietl, Hof, Martin, Magistretti, & Palacios, 1990). When further work was done to understand the role of PACAP and VIP in synaptic transmission within the hippocampus, it was found that VIP enhanced the amplitude of excitatory post synaptic currents (EPSCs) evoked in CA1 neurons mediated via a cAMP/PKA signaling, suggesting that VIP may influence long-term synaptic plasticity (Ciranna & Cavallaro, 2003).

VIP produced in neurons has a definite impact on the function of neuroendocrinological systems and VIP produced by lymphocytes similarly produces profound effects on the immune system, however VIP's role in the junction of these systems has yet to be explored. The effect of VIP's production by hematopoietic cells on the central nervous system requires further investigation.

Implications of VIP in neurological behavior

The suprachiasmatic nucleus (SCN) is the part of the CNS most responsible for circadian rhythms (Colwell et al., 2003). Intrinsically photosensitive retinal ganglion cells (ipRGC) function as photoreceptors that respond directly to environmental cues of light and dark, changing their voltages in response to light. ipRGC axons make up the retinohypothalamic tract (RHT) and communicate directly with the SCN via voltage transmission and melanopsin production through the optic nerve, without having to interact with rods and cones (Berson, 2003). The RHT co-produces the neurotransmitters glutamate and PACAP that are then sent to the SCN to influence phase response (Hannibal, Moller, Ottersen, & Fahrenkrug, 2000). VIP and its related neuropeptide, peptide histadine

isoleucine (PHI), are both produced in the SCN where they aid in cell-to-cell communication (Colwell et al., 2003). Previous studies established that the null mutation of the $VPAC_2$ receptor for VIP and PACAP (*Vipr2*$^{-/-}$) in mice resulted in irregular circadian rhythms of rest and activity behavior. In addition, *Vipr2*$^{-/-}$ mice exhibited impaired production of core clock genes mPer1 and mPer2 (Harmar et al., 2002). Without the mPer1 and mPer2 genes, mice suffer from disrupted locomotor activity rhythms during extended exposure to constant darkness (Bae et al., 2001). To further investigate the functional role of VIP *in vivo,* the Waschek group at the University of California in Los Angeles generated a murine model whose VIP and PHI genes were disrupted. Waschek has shown that VIP-KO mice displayed abnormalities in circadian behavior, with mice housed in constant darkness having a 8-hour earlier onset of activity compared with WT mice. The VIP-KO mice, lacking both the VIP and Phi proteins, demonstrated a loss of coherence and precision of the circadian locomotor activity. Their findings revealed that the VIP and PHI peptides are important in the generation of circadian oscillations and normal circadian entrainment to light (Colwell et al., 2003). Aton et al. pursued further studies to analyze behavior in constant darkness and firing rates of individual neurons from *Vip*$^{-/-}$, *Vipr2*$^{-/-}$, and WT SCN. They determined that many SCN neurons required VIP for rhythmicity, whereas others required it for synchrony. $VPAC_2$ receptor agonists were used to restore synchronization among SCN neurons in VIP deficient mice and showed that VIP signaling is imperative in the synchronization of firing rate between SCN neurons (Aton, Colwell, Harmar, Waschek, & Herzog, 2005). Our lab has used his same VIP deficient mice to demonstrate increased levels of immune activation and function, as noted above. In the current studies we have

used these mice as a source for creating radiation chimeras in which either the blood system or the nervous system was deficient in VIP.

Depressive behavior has been modeled in mice using paradigms such as the forced swim test and sucrose preference test. The forced swim test (FST) was devised in 1977 as a screen for depression like behavior in mice and other animal models (Porsolt, Bertin, & Jalfre, 1977). In 2007, Hashimoto et al. showed that PACAP and its receptor, PAC1, are associated with schizophrenia. In a study of 804 patients with schizophrenia, the group demonstrated that certain polymorphisms in the genes for PACAP and PAC1 are associated with schizophrenia. Further investigations with a murine model unveiled that PACAP knockout mice (PACAP$^{-/-}$) exhibited high initial levels of locomotor activity that was only restored with the administration of an atypical antidepressant (R. Hashimoto et al., 2007a). These findings led to further experimentation with PACAP$^{-/-}$ mice in forced swim tests where deficient mice exhibited significantly greater duration of immobility than wild type mice (H. Hashimoto et al., 2009). Because VIP and PACAP both share 67% of their peptide sequence, both peptides play similar and important roles in the control of immunity and behavior (Harmar et al., 2012).

In addition to exhibiting altered circadian rhythms, and increased immobility, VIP-KO and related mice express select cognitive deficits. Recollection of fear responses is a measure of cognitive abilities in murine models. VIP plays an important role in regulating learning and memory functions in the hippocampus, cortex, and amygdala (D. Chaudhury, D. H. Loh, J. M. Dragich, A. Hagopian, & C. S. Colwell, 2008). Chaudhury et al. found that VIP-deficient mice exhibited a significant reduction in recall when tested 48-hours or longer after training of fear-conditioned behavior. Wild type and VIP-KO mice were exposed to

foot shocks of a range of intensities (0.1 to 1 mA) and the overall freezing response to the shock was lower in VIP-KO mice compared to WT controls. Investigators concluded that the loss of VIP impaired recall of contextual fear conditioning.

Hematopoietic cells localize in the central nervous system

Glial cells are closely placed with neurons and play a supportive role in the central nervous system (Angulo, Kozlov, Charpak, & Audinat, 2004). Glial cells are modulators of synaptic function that aid in neurotransmission regulation and support the maintenance of synapse morphology, making glial cells vital in modulation of neuronal activity (Auld & Robitaille, 2003). A particular type of glial cell, the microglia, is considered to be a macrophage-like cell in the central nervous system (Kim & de Vellis, 2005). In particular, microglial cells are continuously monitoring CNS parenchyma for an alteration in homeostasis. Researchers have determined that microglia derive from monocytes and blood-born precursors, maintaining the role of these glial cells in immunological protection of neuronal activity. In addition to regulating inflammation-associated neurogenesis, microglia also play a role in neurogenesis when activated by certain T helper cell cytokines (Hanisch & Kettenmann, 2007). Microglia play an important role in regulating immunological processes within the central nervous system, but may often be aided by immune cells being able to travel from the peripheries across the blood brain barrier into the CNS.

The brain blood barrier (BBB) is a multi-layer system in place to protect the central nervous system from peripheral circulation (Banks & Erickson, 2010). Made up primarily of the capillary bed of the CNS and the choroid plexus, the BBB is an integral component of

the neuroimmune axis and serves to regulate the interactions between the immune system and the CNS. The neurons, microglia, astrocytes, pericytes, and cells that make up the BBB communicate amongst each other to influence the function of the BBB (Neuwelt et al., 2008). More so, the BBB has the ability to secrete substances related to the neuroimmune system, including cytokines, prostaglandins, and nitric oxide (Fabry et al., 1993; Mándi et al., 1997; McGuire et al., 2003; Reyes, Fabry, & Coe, 1999). Epithelial cells of the choroid plexus localized in the ventricles of the brain form a diffusion barrier called the blood-cerebrospinal fluid barrier (BCSFB). In addition, endothelial cells of the leptomeningeal microvessels at the surface of the brain and spinal cord form a distinctive diffusion barrier called the blood-leptomeningeal barrier (BLMB) which goes on to form a BCSFB (Engelhardt & Ransohoff, 2012). Diapedesis is the process by which immune cells cross the BBB (Banks & Erickson, 2010). This mechanism has been primarily studied with T-cells in an experimental autoimmune encephalomyelitis (EAE) model and the main entry for leukocytes into the CNS is through postcapillary venules (Engelhardt, 2008). Due to the dual nature of the BBB, immunosurveillance of the CNS requires immune cells in the peripheral blood stream to either cross the endothelial BBB or BLMB or across the BCSFB in the absence of neuroinflammation. Research indicates that T cell migration across the BLMB is more efficient, as intravenously injected fluorescently labeled T cells were detectable in brain leptomeningeal spaces after 2 hours (Carrithers, Visintin, Kang, & Janeway, 2000). Due to specialized structure of the central nervous system parenchyma, there's migration of immune cells across the BBB or BCSFB, followed by progression across the glia limitans into the CNS parenchyma during neuroinflammation. In healthy individuals, CD4+ memory T cells enter the CSF in a regulated manner via the BCSFB to

execute immune surveillance of the CNS (Engelhardt & Ransohoff, 2012). Therefore, with all of this established research done in murine models by Engeldhardt and colleagues, it has been established that immune cells cross the BBB, providing evidence that bone marrow cells will be able to cross the BCSFB. This was further reinforced by the 2012 work of Hasegawa-Ishii et al. where the researchers highlighted immune cells interacting with brain parenchyma.

Bone marrow transplant cells localize in the brain adjacent to attachments of the choroid plexus (Hasegawa-Ishii et al., 2013). Mice whose hematopoietic systems reconstituted with bone marrow cells derived from GFP-transgenic mice show the presence of GFP+ microglia within the brain parenchyma in discrete brain regions from 1 to 4 months after BMT. In these experiments the bone marrow donor and transplant recipient were genetically identical (syngeneic transplants) except for the presence of the GFP transgene in the former group. In addition, quantitative light and electron microscopic immunocytochemistry studies of bone marrow derived cells in the CNS in radiation bone marrow chimeras show the CNS is consistently regulated by a T lymphocytes and monocytes. Monocytes of the meninges and perivascular areas are continuously reproduced and replaced by hematogenous cells under normal conditions (Lassmann, Schmied, Vass, & Hickey, 1993).

Although it has been established that hematopoietic bone-marrow-derived cells have the ability to cross the blood brain barrier and that the progeny of intravenously injected bone marrow cells make their way into the brain, the effect of hematopoietic cells on neurological behavior has yet to be explored. VIP has been noted to effect brain function

and immune regulation but the peptide's role in the bidirectional network and where the two systems work together, has yet to be explored. We studied the effects of VIP derived from hematopoietic cells on behavior to understand the importance of the neuropeptide in the bidirectional communication between the immune and central nervous systems. Because we have previously published work highlighting the significance of VIP in murine transplant models, we were interested in using radiation chimeras as a model to control immunologically produced VIP and its effect on neuronal activity. VIP-KO mice were used as a source for creating radiation chimeras in which the blood system and/or the nervous system was deficient in VIP. Only female mice were used in these experiments and while there are notable differences in the hormonal cycles among male and female murine models, investigators have determined that the estrous cycle of female mice does not lead to a significant difference in behavioral effects between C57BL/6J male and female mice (Meziane, Ouagazzal, Aubert, Wietrzych, & Krezel, 2007). Meziane et al. reported strain differences between C57BL/6J and BALB/cByJ female mice in the open field, tail flick, and tail suspension tests, but the behavior compared between genders of the same strain was not significantly different.

Therefore, two non-transplanted groups (WT and VIP-KO) and four radiation chimera groups (wild type mice engrafted with either WT or VIP-KO bone marrow-derived cells and VIP-KO mice engrafted with either WT or VIP-KO bone marrow-derived cells) were tested. We hypothesized that behaviors of VIP KO mice will be changed to resemble wild type mice in radiation chimeras engrafted with hematopoietic cells from WT mice and the behavior of wild type mice will be changed to resemble VIP KO mice in radiation chimeras of wild type mice engrafted with hematopoietic cells from VIP KO mice.

Our hypothesis determining bone marrow-derived cells influencing behavior was supported. VIP produced by blood cells are important in maintaining psychomotor activation levels in female mice.

METHODS

Mice

Two to three months old female C57BL/6j (B6 strain) VIP wild type mice were used as non-transplanted WT controls (n=8) or transplanted WT recipients (n=16). Similarly, 2 to 3 month old female B6 VIP/PHI-KO mice (op cit.) were used as either non-transplanted VIP-KO controls (n=8) or transplanted VIP-KO recipients (n=16) (Colwell et al., 2003). Syngeneic female littermates were used as bone marrow donors. Procedures conformed to the Guide for the Care and Use of Laboratory Animals, and were approved by the Emory University Institutional Animal Care and Use Committee. Transplants were conducted within the Waller Lab and unless otherwise noted, the Emory University School of Medicine Rodent Behavioral Core Facility conducted behavioral tasks. Both animal housing rooms used for transplant and behavioral testing were managed by the Division of Animal Resources at Emory University, and were therefore kept under constant temperature, humidity, and light cycle. Mice were entrained to the standard 12:12 light-dark cycle with lights on at 7 AM and off on 7 PM.

Bone Marrow-Derived Donor Preparation

Bone marrow cells (BMC) were harvested to create chimeric mice with bone marrow-derived cells from VIP-KO donors or WT donors. Femora and tibia were obtained from VIP-

KO and WT mice, cutting off the end of each bone, and flushing out the bone marrow with PBS supplemented with 2% fetal bovine serum (FBS). BMC were passed through a nylon mesh cell strainer, pelleted, re-suspended in red cell lysis buffer, and then washed with FBS-free PBS.

Preparing the Radiation Chimeras - Hematopoietic Cell Transplantation

On day -1, 16 WT and 16 VIP-KO mice were irradiated with two fractions of 5.5 Gy for a total of 11 Gy. On day 0, 8 WT and 8 VIP-KO irradiated mice each received 200 uL of 3.5 X 10^6 BMC from WT donor mice via intra-venous tail injection. Similarly on day 0, 8 WT and 8 VIP-KO irradiated mice each received 200 uL of 3.5 X 10^6 BMC from VIP-KO donor mice via intra-venous tail injection. Wild type recipient mice receiving WT cells (WT → WT), wild type recipient mice receiving VIP-KO cells (KO → WT), VIP-KO mice receiving VIP-KO cells (KO → KO), and VIP-KO mice receiving WT cells (WT → KO), were all monitored for signs of severe infection including posture, activity, and weight loss 30 days post-transplant.

Behavioral Testing:

As per the standard operating procedures of the Rodent Behavioral Core, representatives of the core provided behavioral testing for all 48 mice (both non-transplanted and transplanted groups) in three behavioral paradigms, in the following order: locomotor activity as a measure of circadian rhythms (10-weeks post radiation), forced swim test (11-weeks post radiation), and contextual fear conditioning (12-weeks post radiation).

Circadian Rhythms

Mice were placed in a plexiglass activity cage (with appropriate clean bedding) equipped with infrared photobeams (San Diego Instruments) for 23 hours. The 4 X 8 photobeam configuration of the locomotor activity system tracked the animal's movements. Software provided with the activity tracking system recorded all beam interruptions, central peripheral activity, ambulation (consecutive beam breaks), and rearing activity (when mouse exclusively stood on hind paws). Food and water were available ad libitum. Data was collected as ambulations per hour for the span of 23 hours. Novelty induced locomotion was measured as total ambulations in the first two hours that the mice were placed in the novel activity cage, from 10:30 to 12:30. Dark phase activity was recorded as the total ambulations in the 12 hours of the dark cycle, from 19:00 to 7:00.

Forced Swim Test

Mice were forced to swim and videotaped for 6 minutes in 20 cm of fresh 25 C water in a 4 L beaker (18 cm diameter). In this test, struggling, swimming, and immobile floating behavior was observed, with immobility considered a "depression-like" phenotype. Because previously published work reported that there was only a significance difference in immobility between wild type and PACAP deficient mice, I exclusively scored the forced swim test videos (recorded in the core) for immobility. A mouse was considered immobile when it was only making movements necessary to remain floating. Subtle movements of feet, tail or head required maintaining the eyes, ears, and nose above the surface of the water were excluded as immobility. Videotapes were scored for total time spent immobile in one-minute bins and then summed for total time of immobility. The mice were then

removed from the water, dried, and returned to their home cage. The cage was placed half-on, half-off a heating pad until the mice were dry.

Contextual Fear Conditioning

Contextual fear conditioning is a fear-conditioning task that measures the ability of a rodent to form and retain an association between an aversive stimulus and environmental cues. The behavioral core facility used a modified protocol based on their standard fear-conditioning paradigm and previously published methods (Dipesh Chaudhury, Dawn H. Loh, Joanna M. Dragich, Arkady Hagopian, & Christopher S. Colwell, 2008) that occurred over a span of two days. On the day of training, mice were introduced to a novel environment (conditioned stimulus, CS) for 3 minutes, were exhibited to a 2-second 0.1 mA foot shock (unconditioned stimulus, US), waited 64 seconds before the next 2-second 0.1 mA foot shock, and then waited a final 64 seconds before being removed from the apparatus. The novel environments were fear-conditioning apparatuses (7” W, 7” D X 12” H, Coulbourn) composed of Plexiglas with a metal shock grid floor, sterilized between sessions. On day 2, the animals were presented with a contextual test, during which the mice were placed in the same Coulbourn fear-conditioning apparatus for a total of 8 minutes, where freezing was measured by the Coulbourn software in one minute time bins. Freezing, as measured by the Coulbourn software, is the absence of movement except for respiration (Curzon, Rustay, & Browman, 2009). Percent freezing as noted in the results, is the time animals spent making minimal movement during the 8 minute testing period. The modified protocol used in these experiments duplicated Chaudhury et al.’s 64-second blocks for the sake of comparison.

Statistical Analysis

Statistical analyses were performed using Graph Pad Prism Software (OS X version 6.00 for Macintosh, Graph Pad, San Diego, California, USA). Differences between comparable groups (non-transplanted WT vs. non-transplanted VIP-KO, KO mice receiving KO cells vs. KO mice receiving WT cells, WT mice receiving WT cells vs. WT mice receiving KO cells) were analyzed using a one-way analysis of variance (ANOVA) test followed by a Tukey's multiple comparison post-hoc analysis. Differences between groups were also analyzed using unpaired two-tailed Student's *t* test. Significance was defined by $p \leq 0.05$.

RESULTS

The behavioral paradigms chosen each measure different behaviors and brain areas. The sequence of testing was designed to allow interpretation of each test independently.

Circadian Rhythms: KO Transplants Increase Locomotor Activity

Non-transplanted VIP-KO mice exhibited less movements during the 23-hour cycle compared to WT mice (Figure 1). Based upon these results we predicted that the transplantation of WT cells into KO mice would result in greater total activity than would transplantation of KO cells into KO mice. Similarly, we predicted that the transplantation of WT cells into WT mice would also result in greater total ambulations than would transplantation of KO cells into WT mice. There was a statistically significant difference between transplanted groups as determined by one-way ANOVA ($F(3,28) = 3.104$, $p = 0.0425$). However, we found that total ambulations for KO mice receiving KO cells were

greater than that of KO mice receiving WT cells (Figure 2). Similarly, total ambulations for WT mice receiving KO cells were greater than that of WT mice receiving WT cells (Figure 3).

When novelty induced locomotion measurements were analyzed, it was determined that ambulations of non-transplanted WT mice were greater than that of non-transplanted KO mice (Figure 4A). Based upon these results we predicted that the transplantation of WT cells into KO mice would result in greater novelty induced locomotion than would transplantation of KO cells into KO mice. Similarly, we predicted that the transplantation of WT cells into WT mice would also result in greater novelty induced locomotion than would transplantation of KO cells into WT mice. It was found that there was a statistically significant difference between transplanted groups as determined by one-way ANOVA ($F(3,28) = 4.093$, $p = 0.0158$), but in line with the findings of total ambulations. We found that novelty induced locomotion for KO mice receiving KO cells were significantly greater than that of KO mice receiving WT cells ($t(14)=2.222$, $p = 0.0433$) (Figure 4B). Similarly, novelty induced locomotion for WT mice receiving KO cells was significantly greater than that of WT mice receiving WT cells ($t(14)=2.620$, $p = 0.0202$) (Figure 4C).

When dark phase movements were analyzed, it was determined that activity of non-transplanted WT mice was significantly greater than that of non-transplanted KO mice ($t(14)=2.335$, $p = 0.0350$) (Figure 5A). Based on these results we predicted that the transplantation of WT cells into KO mice would result in greater dark phase activity than would transplantation of KO cells into KO mice. Similarly, we predicted that the transplantation of WT cells into WT mice would also result in greater dark phase activity than would transplantation of KO cells into WT mice. However, results aligned with those

of total ambulations and novelty induced locomotion. There was a statistically significant difference between transplanted groups as determined by one-way ANOVA ($F(3,28) = 10.70$, $p < 0.0001$), and a post hoc analysis using Tukey's multiple comparison test noted there was a significant difference between WT mice receiving a KO transplant and WT mice receiving a WT transplant (Figure 5C). Dark phase activity between KO transplant mice was comparable (Figure 5B).

Forced Swim Test: Time Spent Immobile.

Next, radiation chimeras were tested in the forced swim test for time spent immobile. First it was determined that non-transplanted KO mice spent less time immobile, and thereby greater time mobile, than non-transplanted WT mice (Figure 6). Based on these results, we predicted that KO mice receiving KO cells would exhibit greater mobility than KO mice receiving WT cells. Similarly, we predicted WT mice receiving KO cells would also exhibit greater mobility than WT mice receiving WT cells. Our hypothesis was supported. There was a statistically significant difference between transplanted groups as determined by one-way ANOVA ($F(3,27) = 4.673$, $p = 0.0094$), and a post hoc analysis by Tukey's multiple comparison test noted there was a significant difference between WT mice receiving a KO transplant and WT mice receiving a WT transplant (Figure 7). Additionally, KO mice receiving KO transplants exhibited less immobility than KO transplants receiving WT transplants (Figure 8).

Contextual Fear Conditioning: *Percent Freezing*.

Lastly, mice were tested for cognitive abilities through contextual fear conditioning. Freeze responses were noted as memory recollection. First, non-transplanted WT mice activity was compared to that of non-transplanted KO mice. Because there was no difference between percent freeze response between the two groups, we predicted there would be little to no difference in percent freeze response between KO mice receiving KO cells and KO mice receiving WT cells or WT mice receiving WT cells and WT mice receiving KO cells (Figure 9A). Our hypothesis was supported and no difference was noted (Figure 9B-C).

DISCUSSION

Previous studies have highlighted the presence of bone marrow derived cells in choroid plexus parenchyma (Hasegawa-Ishii et al., 2013). In addition, hematopoietic-lineage cells of bone marrow origin have shown to replace meningeal and perivascular monocytes under normal conditions (Lassmann et al., 1993). Previous studies have established that VIP deficient mice exhibit altered circadian rhythm firing, decreased mobility, and reduced cognitive behavior (Dipesh Chaudhury et al., 2008; Colwell et al., 2003; H. Hashimoto et al., 2009). Based on these investigations we chose to study the behavior of VIP-KO murine radiation chimeras whose hematopoietic systems had been replaced with that of VIP-producing WT bone marrow-derived cells. We found that murine radiation chimera transplanted with VIP-KO hematopoietic cells exhibit increased locomotor activity, as seen during circadian rhythm testing (Figure 4). The role for bone marrow-derived cells in influencing behavior was further reinforced by the results of the

forced swim test. After seeing non-transplanted VIP-KO mice exhibit greater mobility than non-transplanted WT mice, we predicted that WT or KO mice receiving hematopoietic stem cells from KO mice would exhibit great activity (Figure 6). This hypothesis was supported when both WT and KO radiation chimeras engrafted with VIP-KO hematopoietic cells exhibited increased activity. These findings are important because they suggest that VIP produced from hematopoietic cells influence brain function (Figure 7-8). Because transplanted mice were compared to transplanted mice with the same genetic profile (WT radiation chimeras having received KO bone marrow were only compared to WT radiation chimeras having received WT bone marrow), any effects of the transplant on behavior were accounted for. Indeed, we did observe decreased locomotor activity (and struggling in forced swim test) in the WT→WT transplanted animals compared to WT animals, suggesting that the transplant procedure did affect behavior likely through effects of the radiation on the brain. However, these effects on locomotor activity and depressive behavior were partially reversed in the KO-WT chimeras. These findings suggest a role for bone marrow-derived blood cells that lack VIP on brain psychomotor function and behavior.

Although it has been established that hematopoietic bone-marrow-derived cells have the ability to cross the blood brain barrier and that the progeny of intravenously injected bone marrow cells make their way into the brain, the effect of HSTCs on neurological behavior has yet to be explored. VIP has been noted to effect brain function and immune regulation but the peptide's role in the bidirectional network, where the two systems work together, has also yet to be explored. Our studies did not only explore the

effects of either VIP or hematopoietic cells on behavior, but also evaluated the impact bidirectional network activities have on central nervous system function.

While circadian rhythm testing and the forced swim test led to statistically significant results, we did not observe an effect of VIP from bone marrow derived cells on contextual fear conditioning testing. Both non-transplanted and transplanted groups exhibited baseline percent fear responses with no differences between comparable groups. Although our design of the contextual fear conditioning paradigm emulated that of Chaudury et al., our non-transplanted WT and VIP-KO mice did not exhibit the same difference in percent fear response (Figure 9). Possible explanations include differences in mouse handling, and confounding factors from subjecting mice to different behavioral assays. The Colwell group allowed mice to run on running wheels as a way of measuring activity time to relate to circadian time. The running wheel may serve as healthy exercise for the mice, influencing other behaviors. Additionally, Chaudury et al. reported a significant difference in percent freezing between WT and VIP deficient mice at 0.1 mA. Therefore, our own protocol followed the published findings and also conducted foot shocks at 0.1 mA. However, the average foot shock used in contextual fear conditioning tests use foot shocks of 0.6 mA, a higher but safer dose (Curzon et al., 2009). It would be worth repeating this portion of the experiment using a higher foot shock dose.

Additionally, the whole-body radiation used in creating the radiation chimeras may have influenced behavior in these experiments. Rola et al. subjected 21-day-old C57BL/6J male mice to whole brain irradiation and assessed three months later. Decreased counts of activated microglia indicate reduced neurogenesis and young mice exhibited deficits in spatial memory in the Morris water maze task, suggesting the radiosensitivity of the

dentate subgranular zone (Rola et al., 2004). Therefore, future experiments may exhibit altered cognitive results if the brains of the radiation-chimeric mice are protected from radiation. Studies have established that when mice heads were protected with lead caps, twice the dosage of radiation was required to produce the same survival curve as that of a mouse receiving full body irradiation (Reinhard, Mirand, Goltz, & Hoffman, 1954).

The greatest difference between Chaudury et al.'s experimentation and our own, however is the gender of the mice used. While their experiments were done with all male mice, ours were done exclusively with female mice of the same strain. In his chapter on sex differences in brain damage and recovery, Stein cites numerous studies highlighting differences from hormonal status playing a role in normal and brain-damaged human and animal subjects (Stein, 2007). Studies citing all female studies are few in comparison to those of all male studies. Female mouse models are often not used for experimentation due to hormonal changes, but as Stein notes, they are important in understanding brain function. Therefore, while it may seem that the gender in this set of experiments is a weakness, it is actually a strength that may support future scientific endeavors for the behavioral immunology community. While there are notable differences in the hormonal cycles among male and female murine models, investigators have determined that the estrous cycle of female mice does not lead to a significant difference in behavioral effects between C57BL/6J male and female mice (Meziane et al., 2007). Meziane et al. reported strain differences between C57BL/6J and BALB/cByJ female mice in the open field, tail flick, and tail suspension tests, but the behavior compared to genders of the same strain was not significantly different.

In order to validate and further test our hypothesis, we plan on repeating the aforementioned experiments. While the results are significant, the claim of radiation chimeras influencing behavior requires repeated results. For the purpose of noting sex differences, all future experiments will include both female and mice radiation chimeras. Additionally as was previously mentioned, it is worth repeating Contextual Fear Conditioning testing with higher, but safe, shock voltages. To further test the effect of radiation chimera on neurological function, we also plan on testing non-transplanted and transplanted murine groups in different behavioral paradigms such as Morris Water Maze and Cued Contextual Fear Conditioning tests. These assays are important in testing mice for cognitive abilities in different paradigms.

To understand the complete impact of radiation chimeras on behavior, further experimentation includes histological analysis of murine brain areas. While it is primitive to say that the absence of VIP from peripheral immune cells is directly impacting the SCN or parts of the cerebral cortex where VIP is produced, this study highlights the significance of repeating these experiments and pursuing further neurological analysis to determine how VIP produced by blood cells is influencing the activation of psychomotor activation levels. As mentioned previously, VIP deficient mice suffer from cognitive deficits and in severe cases VIP-KO mice emulate cognitive losses comparable to that of Alzheimer's patients (Delgado & Ganea, 2003; R. Hashimoto et al., 2007b). PACAP deficient mice exhibit schizophrenic like behavior (R. Hashimoto et al., 2007b). Investigators have described the potential therapeutic properties of VIP in treatment of disorders such as Alzheimer's disease, Parkinson's disease, and Autism Spectrum Disorders (White, Ji, Cai, Maudsley, & Martin, 2010). While these findings differ from what we found in our studies, further

investigations may aid in finding drug treatments that may modulate the activity of hematopoietic and immune cells to influence neurological function. With the aid of immunohistochemical testing and analysis of brain slices, these speculations can be tested experimentally.

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FIGURES

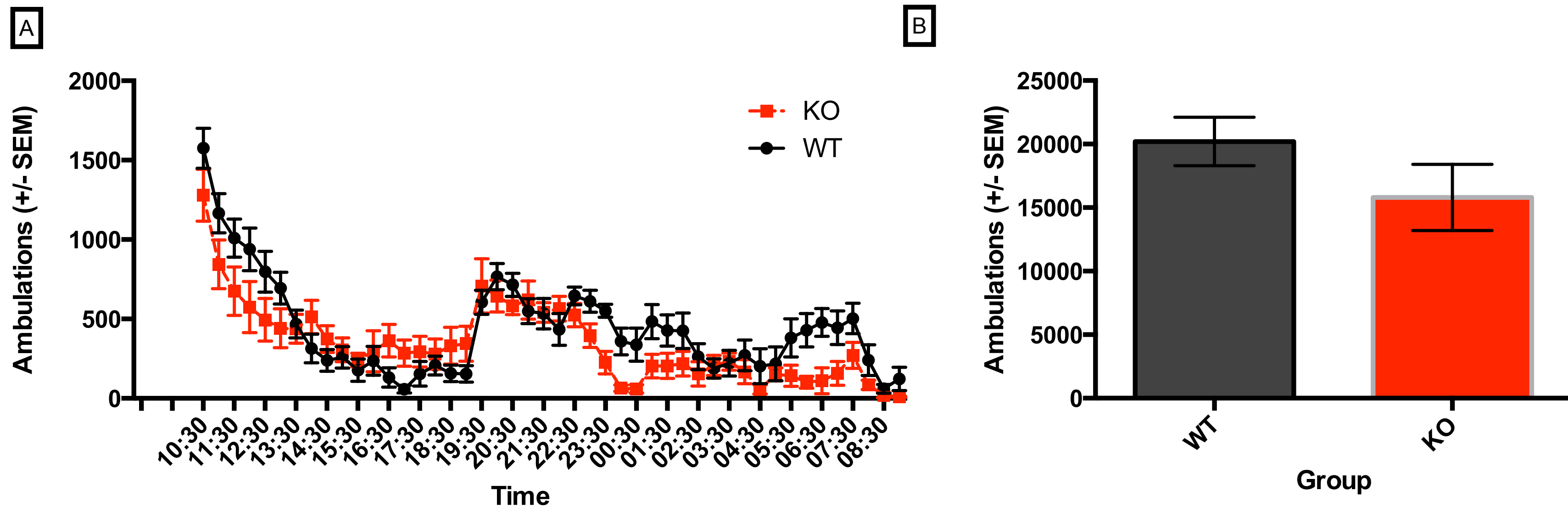

Figure 1: **Total Ambulations of Non-Transplanted Groups.** VIP-KO and WT mice locomotor activity was tracked by breaks in laser beams and analyzed by computer software. **(A)** Mean ambulations (n=8) were noted per hour for a period of 23-hours. **(B)** Total ambulations during the 23-hour period were summed and recorded.

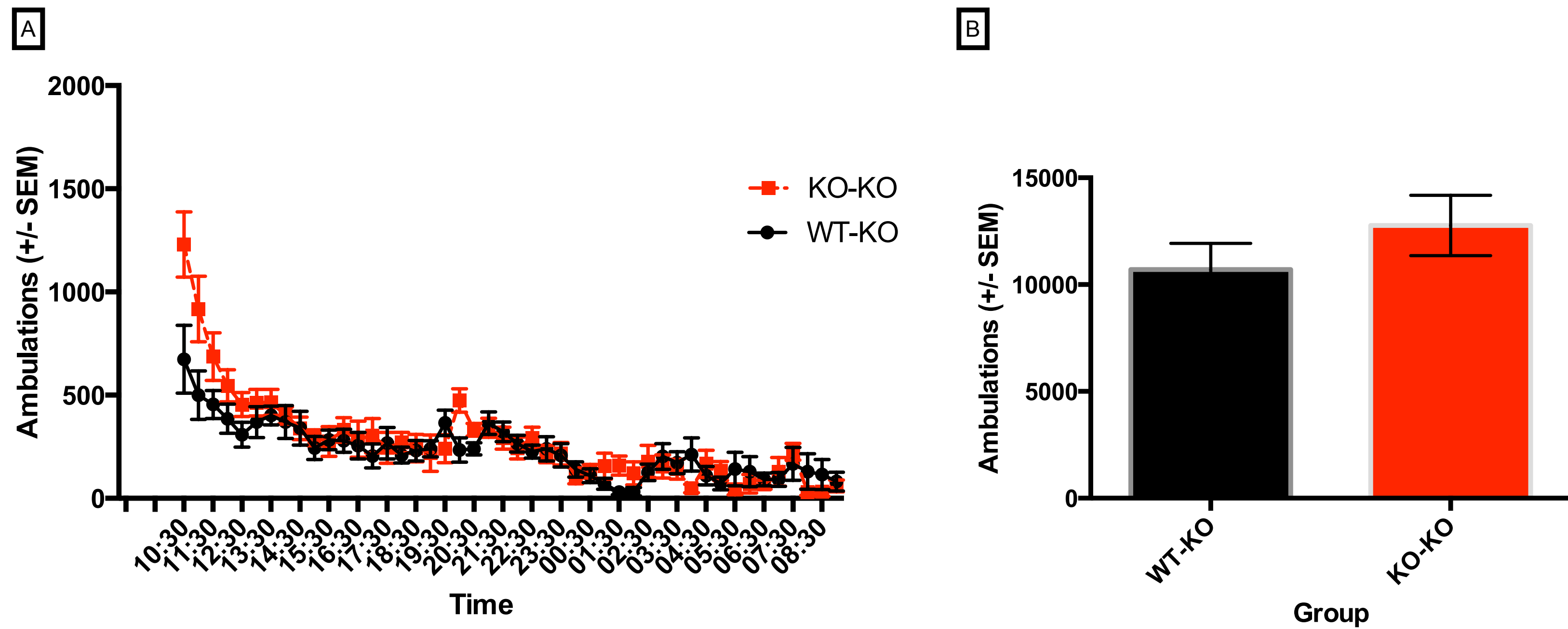

Figure 2: **Total Ambulations of KO Transplanted Groups.** The locomotor activity of VIP-KO radiation chimeras receiving either KO bone marrow (KO-KO) or WT bone marrow (WT-KO) was tracked by breaks in laser beams and analyzed by computer software. **(A)** Mean ambulations were noted per hour for a period of 23-hours. **(B)** Total ambulations during the 23-hour period were summed and recorded. N=8, per group.

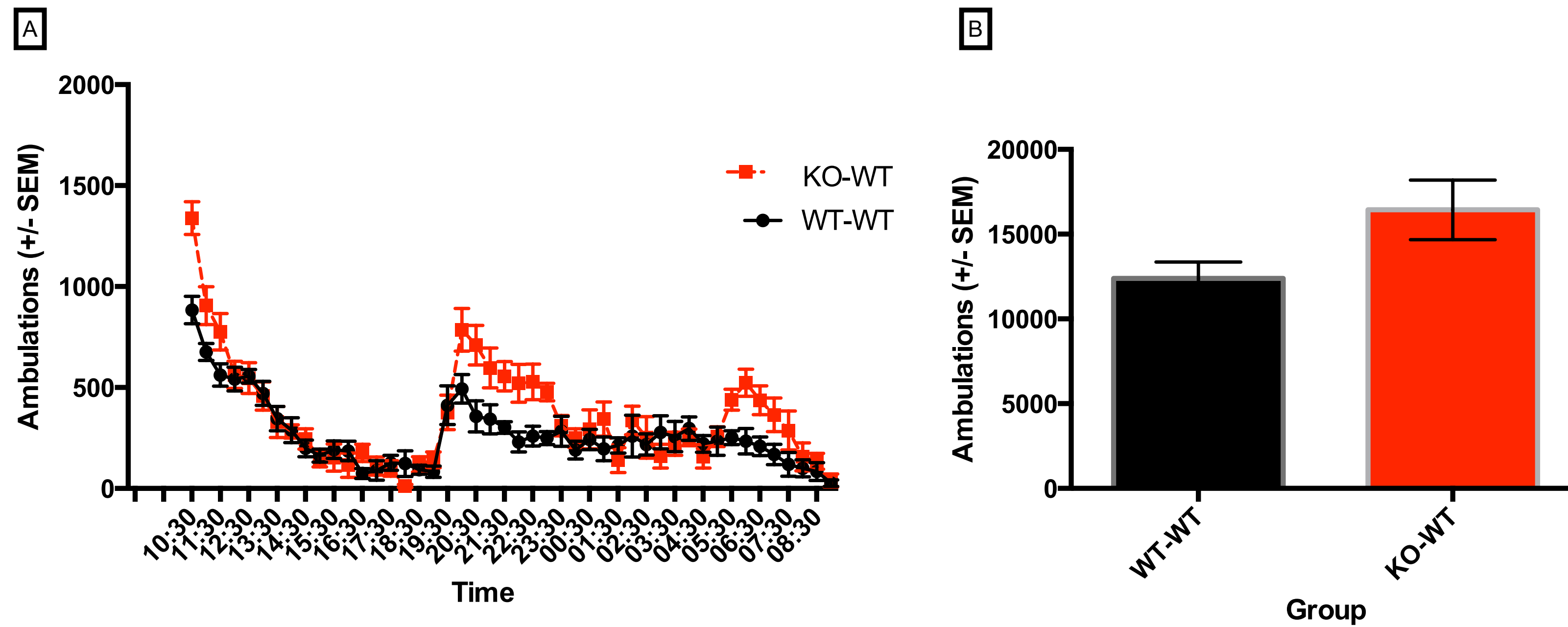

Figure 3: **Total Ambulations of WT Transplanted Groups.** The locomotor activity of WT radiation chimeras receiving either KO bone marrow (KO-WT) or WT bone marrow (WT-WT) was tracked by breaks in laser beams and analyzed by computer software. **(A)** Mean ambulations were noted per hour for a period of 23-hours. **(B)** Total ambulations during the 23-hour period were summed and recorded. N=8, per group.

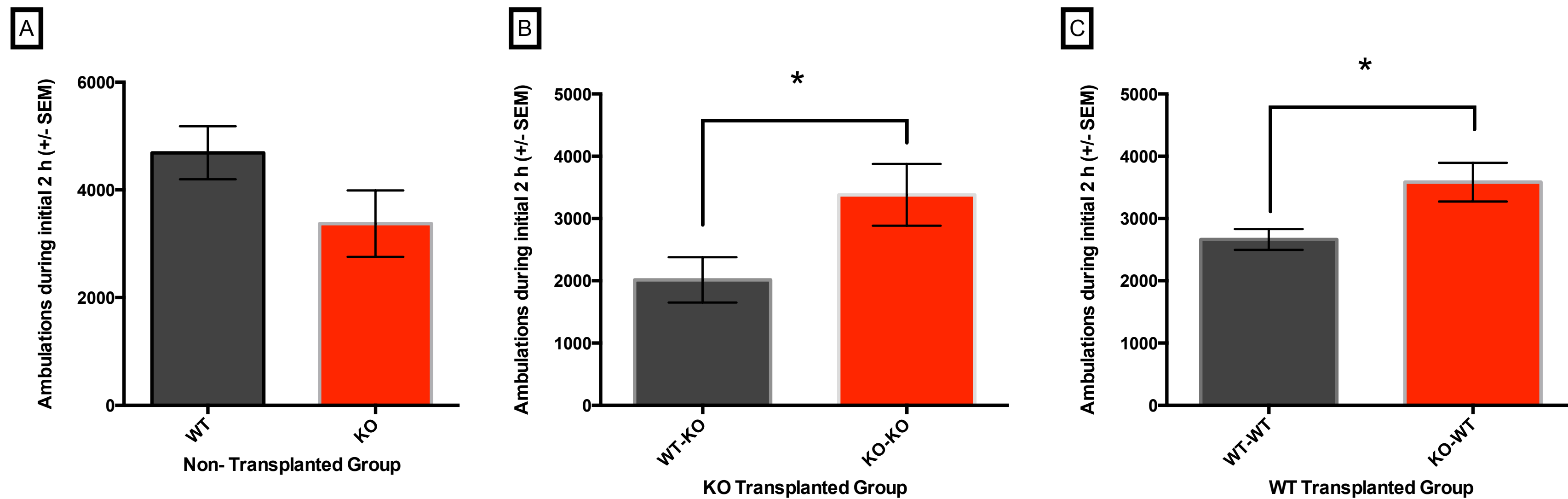

Figure 4: **Novelty Induced Locomotion of Non-Transplanted and Transplanted Groups.** Novelty induced locomotion was measured as total ambulations in the first two hours that the mice were placed in the novel activity cage, from 10:30 to 12:30, during locomotor activity testing. **(A)** Mean ambulations for non-transplanted groups, WT and VIP-KO mice, were recorded. **(B)** Mean ambulations for KO radiation chimeras receiving either KO bone marrow (KO-KO) or WT bone marrow (WT-KO) were recorded. Significant difference was noted ($t(14)=2.222$, $p = 0.0433$). **(C)** Mean ambulations for WT radiation chimeras receiving either KO bone marrow (KO-WT) or WT bone marrow (WT-WT) were recorded. A significant difference was noted ($t(14)=2.620$, $p = 0.0202$). N=8, per group.

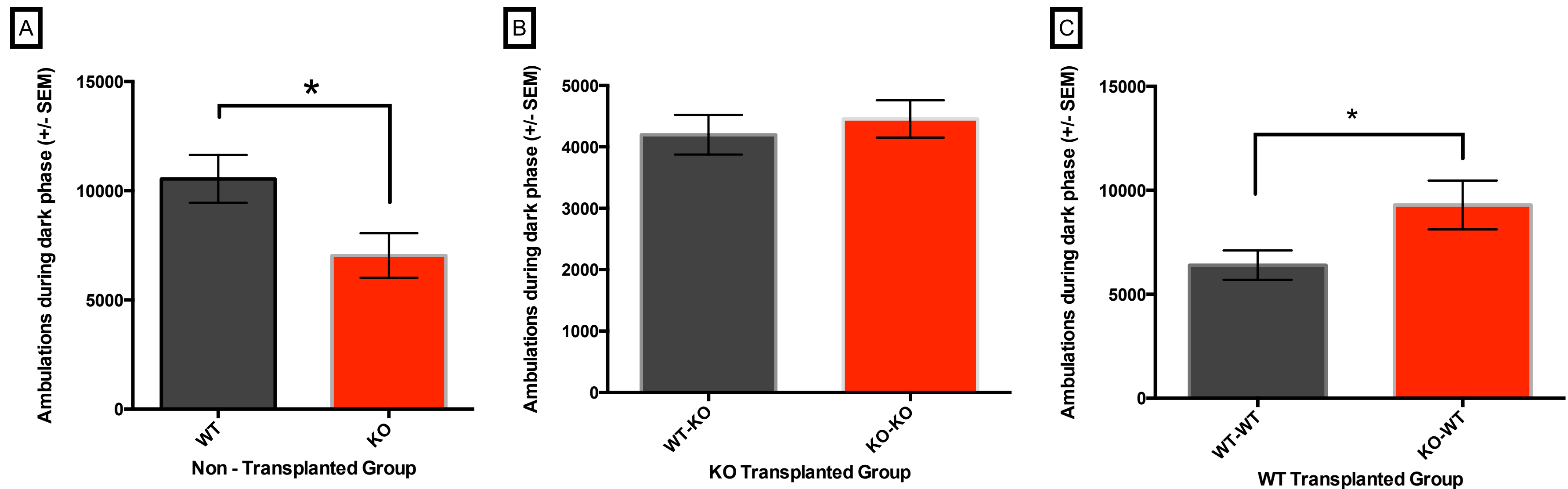

Figure 5: **Dark Phase Activity of Non-Transplanted and Transplanted Groups.** Dark phase activity was recorded as the total ambulations in the 12 hours of the dark cycle, from 19:00 to 7:00, during locomotor activity testing. **(A)** Mean ambulations for non-transplanted groups, WT and VIP-KO mice, were recorded. A significant difference was noted ($t(14)=2.335$, $p = 0.0350$). **(B)** Mean ambulations for KO radiation chimeras receiving either KO bone marrow (KO-KO) or WT bone marrow (WT-KO) were recorded. **(C)** Mean ambulations for WT radiation chimeras receiving either KO bone marrow (KO-WT) or WT bone marrow (WT-WT) were recorded. A post hoc analysis using Tukey's multiple comparison test noted there was a significant difference after a one-way ANOVA noted a statistically significant difference between transplanted groups ($F(3,28) = 10.70$, $p < 0.0001$). N=8, per group.

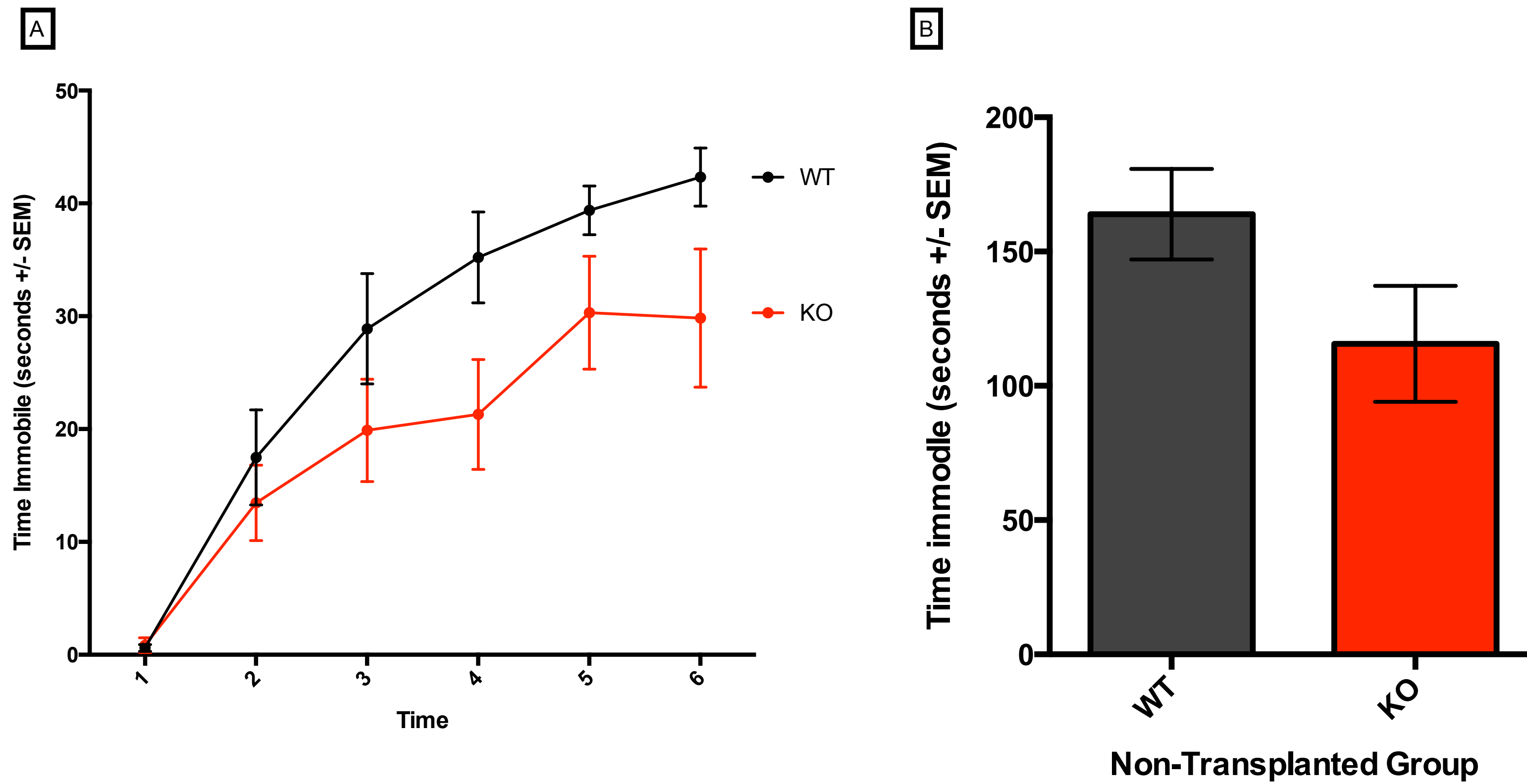

Figure 6: **Forced Swim Test of Non-Transplanted Groups.** WT and VIP-KO mice were tested for total time spent immobile. **(A)** Mean immobility per minute was recorded. **(B)** Total time spent immobile was summed for each group. N=8, per group.

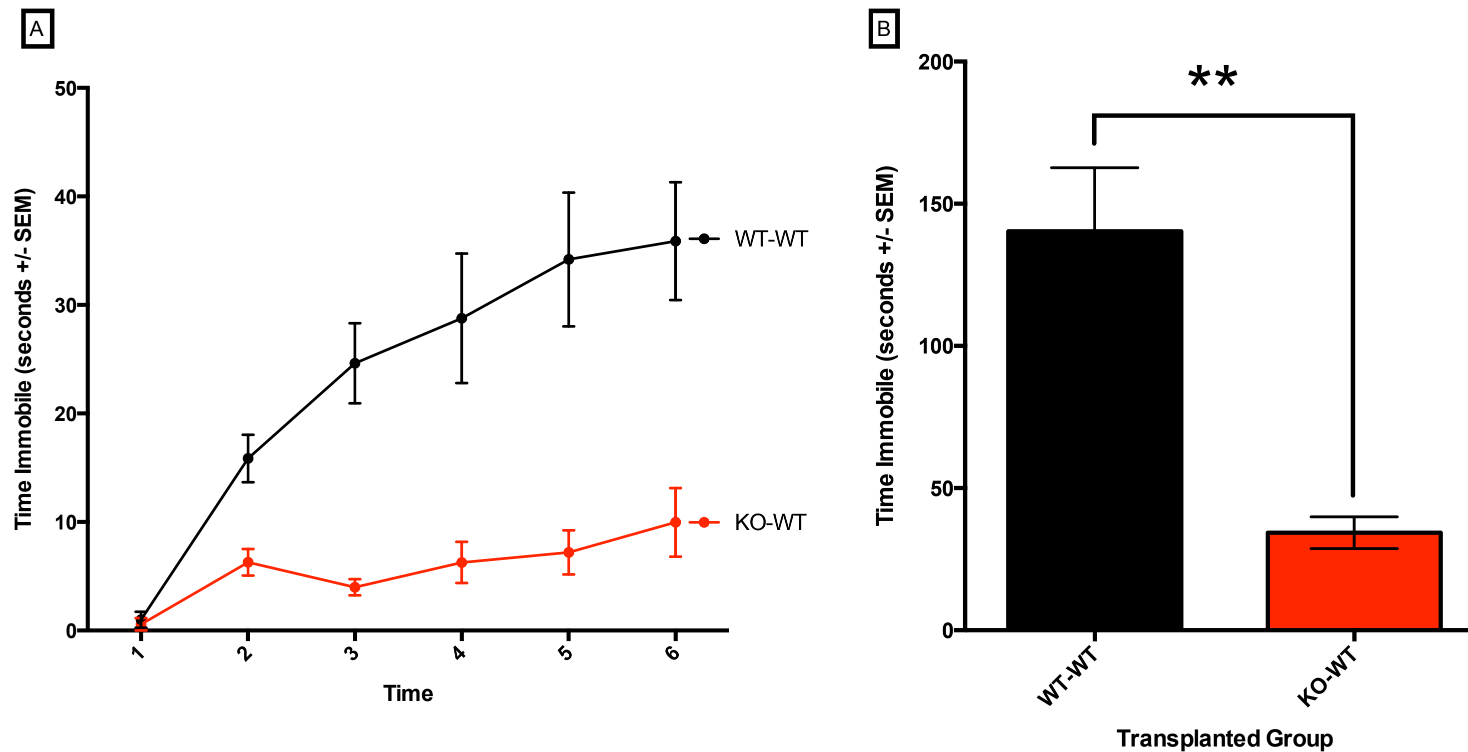

Figure 7: **Forced Swim Test of WT Transplanted Groups.** WT radiation chimeras receiving either KO bone marrow (KO-WT) or WT bone marrow (WT-WT) were tested for total time spent immobile. **(A)** Mean immobility per minute was recorded. **(B)** Total time spent immobile was summed for each group. There was a statistically significant difference between transplanted groups as determined by one-way ANOVA ($F(3,27) = 4.673$, $p = 0.0094$), and a post hoc analysis by Tukey's multiple comparison test noted there was a significant difference between WT mice receiving a KO transplant and WT mice receiving a WT transplant. N=8, per group.

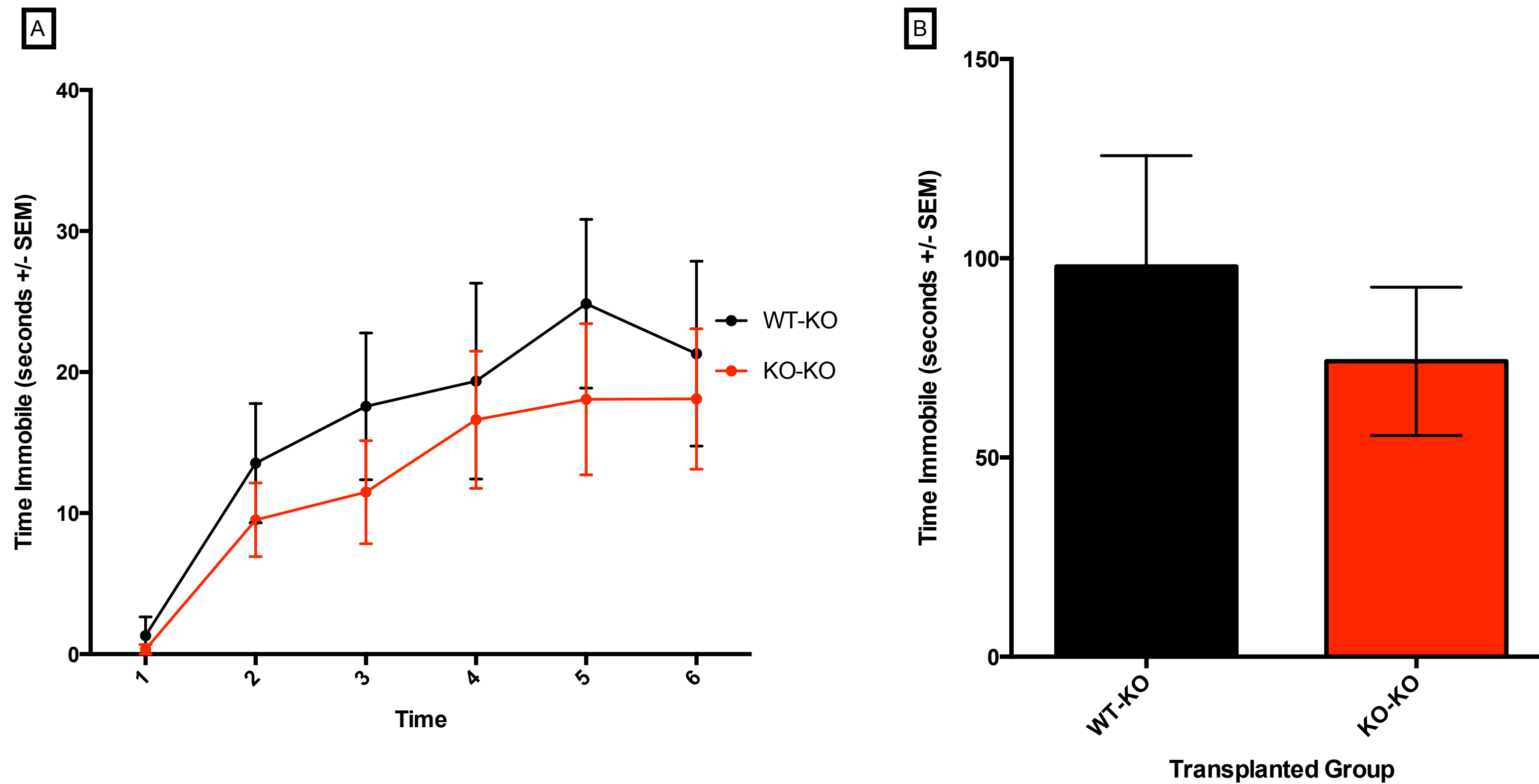

Figure 8: **Forced Swim Test of KO Transplanted Groups.** KO radiation chimeras receiving either KO bone marrow (KO-KO) or WT bone marrow (WT-KO) were tested for total time spent immobile. **(A)** Mean immobility per minute was recorded. **(B)** Total time spent immobile was summed for each group. N=8, per group.

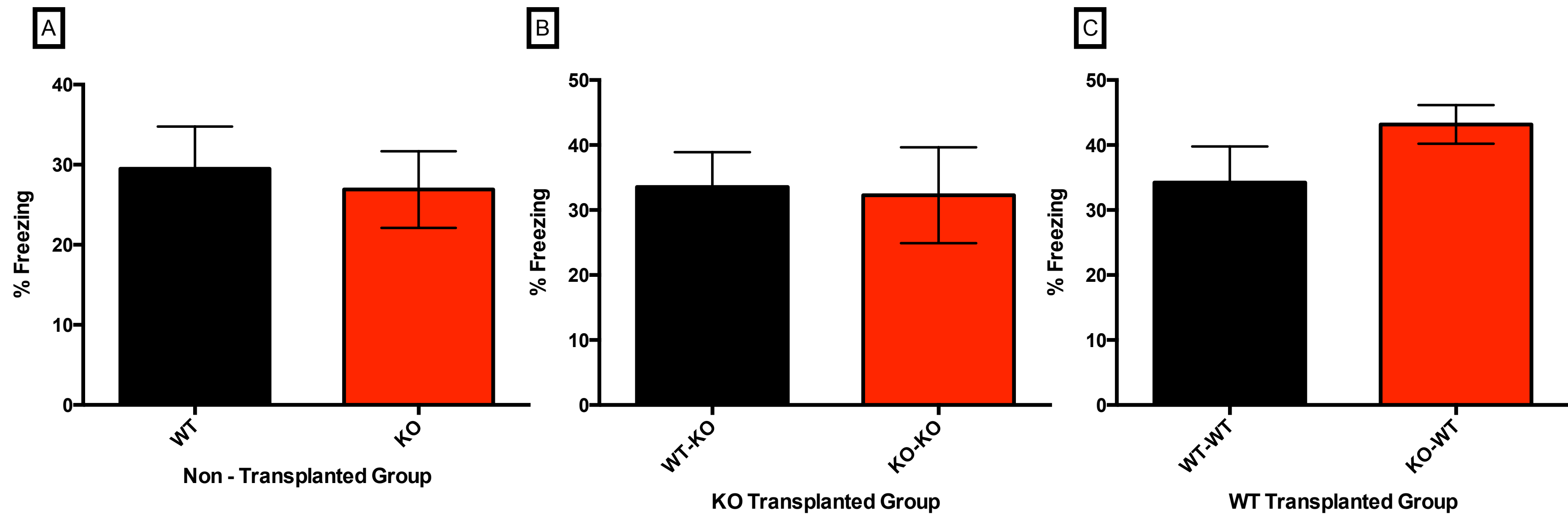

Figure 9: **Contextual Fear Conditioning for Non-Transplanted and Transplanted Groups.** Percent freeze response, total time out of 8 minutes mice spent making minimal movements, was recorded. **(A)** Mean (+/- SEM) percent freeze response for non-transplanted groups, WT and VIP-KO mice, were recorded. **(B)** Mean (+/- SEM) percent freeze response for KO radiation chimeras receiving either KO bone marrow (KO-KO) or WT bone marrow (WT-KO) were recorded. **(C)** Mean (+/- SEM) percent freeze response for WT radiation chimeras receiving either KO bone marrow (KO-WT) or WT bone marrow (WT-WT) were recorded. N=8, per group.