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Controllable and uncontrollable stress differentially impact pathogenicity and survival in a mouse model of viral encephalitis

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Abstract

Intranasal instillation of vesicular stomatitis virus (VSV) into mice given controllable stress (modeled by escapable foot shock, ES) resulted in enhanced pathogenicity and decreased survival relative to infected mice given uncontrollable stress (modeled by inescapable foot shock, IS) and non-shocked control mice. Survival likely reflected differential cytokine gene expression that may have been regulated by miR146a, a predicted stress-responsive upstream regulator. Controllability also enhanced the accumulation of brain T resident memory cells that persisted long after viral clearance. The unexpected facilitatory effect of ES on antiviral neuroimmune responses and pathogenicity may arise from differential immunoactivating and immunosuppressive effects of uncontrollable and controllable stress.

Graphical abstract

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Keywords

Vesicular stomatitis virus; Neuroinflammation; Stressor Controllability; Cytokine; MicroRNA; Innate Immunity

1. Introduction

Stress-related immune dysregulation is increasingly being linked to a variety of health risks (especially those associated with negative emotions (Kiecolt-Glaser et al., 2002; Nakata, 2012)), and to increased neuroinflammation (Angelidou et al., 2012; Garate et al., 2013) and neural pathogenesis (Barnum et al., 2012; Frischer et al., 2009; Garate et al., 2014; Karagkouni et al., 2013; Zhao et al., 2011). However, a significant issue in assessing the potential effects of stress on illness is that the effects of stress are complex and are not predictable based on simply experiencing a stressful event or the elicitation of a stress response. That is, even though stress can have a significant, negative impact on health (Adamac and Shallow, 1993; Pynoos et al., 1996; Shalev, 2000; Van den Berg et al., 1998; Van Dijken et al., 1992), stressors are most often encountered without producing persisting or pathological changes. The outcomes of stress can vary with the characteristics of the stressful event including its controllability (Bolstad and Zinbarg, 1997; Foa et al., 1992), predictability (Abbott et al., 1984; Adell et al., 1988), duration, and intensity (Buydens-Branchey et al., 1990; Natelson, 2004). An individual's relative resilience and vulnerability is also important (Yehuda et al., 2006).

There are limited data suggesting that stressor controllability can differentially impact the immune system with consequences for health. For example, uncontrollable tail-shock stress in mice increased allergen-induced lung inflammation and histopathological changes whereas inflammation and lung pathology were significantly attenuated in mice that could control tail-shock (Deshmukh et al., 2010). There is also evidence that stress-induced suppression of the immune response can facilitate infection, e.g., restraint stress can increase the pathogenicity of herpes simplex virus type 1 (Ashcraft and Bonneau, 2008; Bowman et al., 1987). How stressor control influences the immune system and ultimately host resistance remains poorly defined though there are indications that the ability of stress to have immunoactivating and immunosuppressive effects may have differential consequences for health.

We have been using a simple animal model based on training with escapable shock (ES) and yoked inescapable shock (IS) to model controllable and uncontrollable stress, respectively (Liu et al., 2003; Sanford et al., 2003a; Sanford et al., 2003b; Tang et al., 2005). In this model, animals receive virtually identical amounts of footshock. We have found that training with ES and IS produce significant differences in several behavioral and neurobiological responses despite virtually identical stress responses (increases in corticosterone (an index of HPA activation) and stress-induced hyperthermia (an increase in core body temperature that parallels the time course of corticosterone (Groenink et al., 1994; Veening et al., 2004))) and fear responses (behavioral freezing, an index of stress-related learning and memory (Blanchard and Blanchard, 1969)). The differences include directionally different changes in

post-stress rapid eye movement (REM) sleep (Sanford et al., 2010; Yang et al., 2010) as well as differential activation of brain stress-regulatory regions (Liu et al., 2009).

In this study, we used this ES-IS model to assess the effects of stressor controllability on the neuroinflammatory response and sickness produced by a pathogenic challenge, vesicular stomatitis virus (VSV) which is a member of the Rhabdoviridae virus family and a well-established mouse model of acute viral encephalitis (Bi et al., 1995; Huneycutt et al., 1993; Huneycutt et al., 1994). VSV neuroinvasion elicits a robust inflammatory response characterized by a mixed infiltrate of blood-derived inflammatory cells (Bi et al., 1995; Steel et al., 2010). Critically, for our studies, VSV has an established time course of progression through the brain, and of onset and recovery from encephalitis. Intranasally administered VSV initially replicates in olfactory receptor neurons and subsequently infects the deeper layers and surrounding cells of the nasal mucosa (Reiss et al., 1998). VSV protein can be detected in the olfactory bulb (OB) on day 1 post infection (PI) and then over the course of the next 6 to 7 days, it travels caudally to infect regions and tracts extending into the brain stem (Huneycutt et al., 1994) with maximal sickness occurring on days 5 to 7 PI (Bi et al., 1995; Huneycutt et al., 1993; Huneycutt et al., 1994). Major structures that show VSV antigen include the septal region, the amygdala, bed nucleus of the stria terminalis, hypothalamus, thalamus and hippocampus, and the dorsal pons including the noradrenergic locus coeruleus and the serotonergic dorsal raphe nucleus (Huneycutt et al., 1994). By day 12 PI, the virus has cleared the brain (Christian et al., 1996) and animals typically recover from infection.

To evaluate the potential effects of controllability on VSV pathogenicity, we examined VSV pathogenesis by monitoring weight and mortality across the time course of infection and we examined immune signaling pathways, chemokine and cytokine responses in brain regions impacted by VSV. We report herein that stressor controllability markedly impacts the ability of the host to limit morbidity and survive infection. These differences appear to be related to how controllability differentially regulates the antiviral immune response in the CNS that ultimately impacts host morbidity and survival.

2. Methods

2.1. Animals

All studies were performed in C57Bl/6J male mice purchased from Charles River Laboratories (Wilmington, MA) and housed in sterile microisolator cages and maintained in our biosafety level 2 animal facility. Sterile food and water were given ad libitum. The colony room was kept on a 12:12 light-dark cycle and ambient temperature was maintained at $24^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$. Throughout the experiments, measures were taken to avoid unnecessary pain and discomfort of the animals. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Experimental Animals and were approved by Eastern Virginia Medical School's Animal Care and Use Committee.

2.2. Behavioral Procedures

After habituation to the footshock and then were VSV infected (non-shocked, NS). Basal mRNA levels were determined from control mice that experienced the shuttlebox but never received footshock and were then mock infected (intranasal PBS, NS/M). Training took place at the same time on 6 consecutive shock training days (ST1-ST6). The mice were allowed to freely explore the shuttlebox for 5 min after which they were presented with 20 foot shocks (0.5 mA, 5.0 sec maximum duration) at 1.0 min intervals. Five min after the last shock, the mice were returned to their home cages. The entire procedure was of approximately 30 min duration and occurred during the fourth hour of the lights on period. The chamber was thoroughly cleaned with diluted alcohol before each training session. After each ST session, the animals were left undisturbed in their home cages until the training session on the following day. After the third ST session, the mice were briefly anesthetized and VSV was administered intranasally as described below. Coulbourn Graphic State software (ver. 2.1) running on a desktop computer was used to control the administration and timing of footshock to ensure that mice in the ES and IS conditions receive the same duration of footshock, and it was also used to obtain a measure of latency to assess escape performance and to estimate duration of footshock the mice received. Footshock was produced via Coulbourn Precision Regulated Animal Shockers (Model E13-14) and administered via grid floors in the shuttleboxes.

2.3. Virus infection and cytokine array profiles

Wild-type VSV-Indiana strain, provided by Dr. Philip Marcus, University of Connecticut, was grown and assayed as previously described (Marvaldi et al., 1977). Virus was grown in confluent monolayers of Vero cells and virus titers determined by standard plaque assays (Sekellick and Marcus, 1986). VSV was introduced into the brain via intranasal application of 5ul/nostril with 5×10^4 PFU VSV (Barna et al., 1996). At the indicated times, mice were euthanized, perfused with cold PBS and either whole brains or discrete brain regions isolated for subsequent analysis. RNA was purified from homogenized brain tissue using the RNeasy Plus Universal Mini Kit (Qiagen, catalogue number 72404) following the manufacture's instructions. Individual RNA samples were pooled and antiviral immune responses or proinflammatory cytokine profiles characterized by real-time PCR using commercial antiviral (Qiagen, catalogue number PAMM-122ZD) or inflammatory cytokine arrays (Qiagen, catalogue number PAMM-0011D), respectively. Brain cytokine mRNA levels from NS/M were used as controls to calculate fold differences in experimental groups. Input RNA was normalized using house-keeping genes provided with each array.

2.4. VSV-N real-time qPCR assay

To assess the presence of VSV, a qPCR assay was established for the detection of the gene encoding the nuclear (N) protein of VSV. Brain RNA (100ng) isolated from the indicated locations was reversed transcribed using 50 μ M random hexamers (Applied Biosystems, catalogue no. S06405), 2.5 mM dNTPs from 100mM dNTP set PCR grade (Invitrogen, catalogue no. 10297-018), 5 $\times$ First Strand M-MLV RT buffer (Ambion, catalogue no. 8704G), M-MLV Reverse Transcriptase at 100units/ μ L (10,000 U, Ambion, catalogue no. AM2044). The reactions were run in a thermocycler at 42 C for 60 minutes, 95 C for 3

minutes to inactivate the enzyme and then cooled to 4 C. Generated cDNAs were stored at -20 C until qPCR assay. qPCR reactions were performed using multiple concentrations of cDNA to insure optimal amplification conditions. Single reactions were prepared for each cDNA concentration using the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific, catalogue no. K0241). Each PCR reaction also included a nuclease free H₂O negative control and a positive control containing cDNA known to express VSV-N at detectable levels. Each reaction consisted of 1 µM forward and reverse primer, SYBR Green/Fluorescein Master Mix, and 1 µL cDNA for a total volume of 25 µL. Real-time qPCR was run on the BioRad CFX96™ Real-Time PCR Detection System. The device was connected via Universal Serial Bus (USB) 2.0 cable to a laptop computer with a Windows 7 Operating System and CFX Manager software Version 3.1. The cycling conditions were 1 cycle of 95 C/15 minutes followed by 40 cycles of amplification (95 C/15 sec, 66 C/20 sec, 72 C/30 sec, 95 C/15 sec, 64 C/20 sec, 72 C/30 sec, 95 C/15 sec, 62 C/20 sec, 72 C/30 sec, 95 C/15 sec, 60 C/20 sec, 72 C/30 sec, 95 C/15 sec, and 39 cycles at 58 C/20 sec, 72C/30, 95 C/15 sec. Primer sequences were as follows: Gapdh (sense): 5' TCT GGA AAG CTG TGC CGT G – 3'; Gapdh (antisense): 5' – CCA GTG AGC TTC CCG TTC AG – 3'; VSV-N (sense): 5' – GAT AGT ACC GGA GGA TTG ACG ACT A- 3'; VSV-N (antisense): 5' – TCA AAC CAT CCG AGC CAT TC– 3'.

2.5. miR-146a Loop-Taqman PCR assay

For reverse transcription (RT), each reaction tube contained 4uL 10X reaction buffer solution, 0.5uL (5U/uL) RNAase inhibitor, 1uL 2.5mM dNTPs, 0.5uL (100U/uL) MMLVRT (all Invitrogen, Carlsbad, CA), 1uL of 2uM RT loop primer (5' - **GTCGTATCCAGTGCAGGGTCCGAGGTATTGCACTGGATACGACAACCCA**-3'), and 20uL of sample material or synthetic miR-146a control (5' - UGAGAACUGAAUCCAUGGGUU-3'). Reactions were annealed for 30 minutes at 16° C, heated to 42° C for 30 minutes, and inactivated for 5 minutes at 85° C. The italicized bases in the RT loop primer constitute the looped section whereas the flanking bold segments are part of the double-stranded stem region, leaving the 5' six nucleotides (AACCCA) free to prime the miR-146a seed sequence. All RT and PCR primers were synthesized by Integrated DNA Technologies (Coralville, IA). Each PCR reaction contained 17.5uM universal forward primer (5' -GTGCAGGGTCCGAGGT-3'), 1uL of 37.5uM reverse miR-146a primer (5' -GCCCGCTGAGAACTGAATTCCATG-3'), 1uL of 5.0uM TaqMan miR-146a probe (5' -6FAM-TCGCACTGGATACGACAACCCATG- BHQ1-3'), 250 mM MgCl₂, 2 units of AmpliTaq GOLD polymerase (Invitrogen), 100 nM dNTPs (each), 20ng cDNA (from total RNA), and 2.5ul 10× GOLD buffer in 25uL volume. A standard curve was included using control miR-146a (10⁶ to 10¹ copies) and all samples were cycled in a BioRad CFX-96 PCR machine programmed to cycle once at 95° C for 10 minutes then followed by 60 cycles of 15 seconds at 95° C and 1 minute at 60° C.

2.6. Cytokine gene expression assessed by individual real-time PCR reactions

cDNAs were generated from the indicated individual brain regions described above for VSV-N and tested at a 1:10 dilution. Real-time qPCR reactions were carried out using the same cycling conditions as described for VSV-N. Each sample was tested processed in triplicate for the gene-of-interest (GOI) and housekeeping gene (HKG) and the averages of

these threshold Ct values were used to calculate HKG to GOI ratios. Hsp90ab1 was used as the housekeeping gene for all GOIs that were analyzed. All amplicons span intronic sequences so as to prevent genomic DNA contamination, in addition to the DNA eliminator solutions that are used in the RNeasy Plus Universal Mini Kit and the RT² first-strand synthesis kits in the RNA isolation and cDNA conversion steps. Primer sequences are provided in Table I.

2.8. Statistical methods

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post-hoc analysis with Tukey-HSD (GraphPad Prism). Assumption checking was done on the residuals of the data using the Shapiro-Wilk Test for Normality and the Levene's Test for Homogeneity of Variance (R Version 3.4.3). Data for those genes selected for further analysis met these assumptions, justifying the parametric analysis of our data through one-way ANOVA. Histograms show the standard error of the mean (SEM) as well as significance levels as indicated. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., non-significant.

2.9. VSV Pathogenesis

12MiceA subset of 9 (10 mice/treatment group) were monitored on the indicated select days for indices of pathogenesis (weight, mobility/paralysis, respiration, fur texture) for one month following completion of our stress/infection paradigm (3 days of ES or IS prior to and after VSV inoculation. Survival rates were also recorded across groups. AT 31 days PI, half 9 the mice/group were euthanized for flow cytometric analysis (see Fig. 8). The remaining mice were examined similarly subjected to a similar analysis on day 38 PI that yielded similar results (data not shown).

2.10. Flow Cytometry

Brains were excised from perfused (30 ml cold PBS) mice, individually homogenized, pooled and then subjected to discontinuous Percoll centrifugation to enrich for microglia and leukocytes as previously described (Steel et al., 2009). (steel 2009) Cells were stained with mAbs to CD11b (clone M1/70) and CD45 (clone 30-F1) to identify microglia and infiltrating leukocytes. Trm cells were identified with mAb to CD103 (clone 2E7). All antibodies were purchased from Biolegend. Acquisition of 20–200,000 events was performed using a Becton Dickinson (San Diego, CA) FACSCalibur using FlowJo software (Tree Star, Ashland, OR). Non-specific binding in the absence of additional Fc block was previously evaluated and did not affect staining patterns. To determine the absolute number of microglia and infiltrating leukocytes in the CNS, a leukocyte gate was first defined based on forward and side scatter characteristics. Preliminary studies verified that these gated cells were CD45⁺ and viable (95%propidium iodide and annexin V negative). The fraction of microglia (CD45^{int}) or infiltrating blood cells (CD45^{high}) within this gate was then used to calculate cell recoveries All gates and quadrants were established with the use of appropriate isotype controls.

3. Results

3.1. Escape

Latency to escape was evaluated on mice receiving 6 days of ES-IS training to assess whether or not the ES mice had learned the escape response (Fig.1, A). A one-way ANOVA across shock training days was significant, $F(5,46) = 2.97$, $p < .05$. Compared to day 1 of training, the mice showed reduced latency to escape on day 3, $p < .05$. However, on days 4-6, latencies actually got longer and were not significantly different from the first day of training. Escape latency also provides an estimate of amount of footshock the mice received, and thus both ES and IS mice were receiving relatively longer shocks on day 3 PI than they were on the day of VSV inoculation.

3.2. Stressor controllability regulates pathogenicity and survival to neuroinvasive VSV

To determine whether differences in stressor controllability are physiologically relevant in the VSV encephalitis model, mice were trained in ES/IS and then infected with a single instillation of intranasal VSV. Mice were then monitored for pathogenesis (weight, mobility/paralysis, respiration, fur texture) and survival for approximately one month following completion of our stress/infection paradigm. It is apparent from Fig. 1B that weight loss was more severe in the ES group relative to the IS and NS cohorts. ES trained mice showed accelerated and sustained weight loss as early as day 2 PI and this became statistically significant on day 8 and remained significant throughout the observation period. Several mice subjected to the ES paradigm also developed hindlimb paralysis indicating caudal migration of VSV and injury to motor neurons in the hindbrain and/or spinal cord. Enhanced pathogenicity of ES trained mice was also associated with significant decrease in survival (C). Although prior studies indicate that controllable stress can be associated with neutral or positive outcomes in a variety of situations (Foa et al., 1992), the present data suggest that, given specific circumstances, controllable stress also can have a profound negative influence on virus induced pathogenesis and host survival.

3.3. Uncontrollable but not controllable stress amplifies innate antiviral signaling pathways

How controllability regulates pathogenicity and host survival is currently unknown. However, survival from VSV encephalitis is known to be critically dependent on innate immunity, particularly the type I IFN (IFN-I) system because in its absence mice can survive only a few days (Muller et al., 1994). The potent antiviral activity of IFN-I likely reflects its ability inhibit VSV replication locally as well spread among synaptically connected neurons (Drokhlyansky et al., 2017). Long-term survival is also dependent on the establishment of chemokine gradients to direct migration of activated T cells and inflammatory cells into the brain parenchyma (Huneycutt et al., 1993). Neuroinvasive VSV is quickly recognized by the innate immune system using intracellular pattern recognition receptors such as Rig-I (retinoic acid-inducible gene-I) (Yoneyama and Fujita, 2009), TLRs (toll like receptors) (Georgel et al., 2007) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) pyrin-containing protein 3 (NLRP3) (Rajan et al., 2011) that recognize viral RNA motifs. Kinetically, VSV neuroinvasion triggers TLR signaling initially (3-12 hrs PI) followed by Rig-I activation by 24 hrs (Ciavarra et al, 2017). Whether stressor controllability alters these early recognition events and the subsequent cytokine response has not been

examined. To test this possibility mice were subjected to either ES or IS training for 3 days, given VSV on the last day of training and the following day the indicated innate signaling pathways assessed in the OB. Fig. 2A demonstrates the overall gene expression pattern with identification of some of es associated with Rig-I and IFN-I signaling were activated by VSV and modulated by the type of stress experienced by the host. Thus, IS was associated with enhanced activation of genes associated with both these pathways (B, C). These include down-stream genes such as chemokines (Cxcl10) and cytokines like *Inf1b* and *Ifna2* (C) that are readily activated by VSV. In contrast, RNA levels were similar in ES mice relative to NS cohorts. These data indicate that relative to uncontrollable stress, controllable stress can attenuate innate signaling pathways that drive neuroinflammation triggered by this neurotropic viral pathogen. These differences in immune activation occur despite the fact that ES and IS animals receive identical amounts of footshock and have virtually identical peripheral stress responses (Maier et al., 1986; Wellman et al., 2015; Yang et al., 2011), though this could differ in infected animals.

3.4. Differential regulation of antiviral immunity is time-dependent

We next examined mice at day 3 PI, a time when VSV replication is well established in the OB. In contrast to the results obtained on day 1 PI, mice given ES now mounted a markedly enhanced cytokine response to VSV (Fig. 3). Ligands for CCR1, CCR2 and CCR5 were dramatically upregulated relative to responses seen in OBs obtained from IS and NS cohorts (B, C and D). This pattern of reactivity also extended to CXC chemokines (E). In some cases the receptor was upregulated as well as their respective chemokine ligands (B). VSV encephalitis is also associated with production of brain proinflammatory cytokines that presumably contribute to the presence of inflammatory cells in the CNS (Ireland and Reiss, 2006). Our array data is consistent with this view where we detected enhanced cytokine gene expression especially for mice subjected to controllable stress (F). For some cytokine receptors, ES was associated with induction of the receptor as well as its ligand(s) (*IL1r2*, F). Although *IL-10* was differentially upregulated by ES (G), we saw little additional evidence for an interaction between stressor controllability and either canonical Th2 cytokines (*IL4*, *IL13*) or receptors for this class of cytokines such as *IL5ra*, *IL6ra*, and *IL6st* (data not shown). Interestingly, a canonical marker for T helper 1 cells (*IFN- γ*) was strongly upregulated by ES, whereas a second Th1 cytokine remained unchanged (lymphotoxin, H). Similarly, *IL-17b* remained near basal levels regardless of stressor type suggesting that Th17 cells were refractory to these stressors. Thus, the ability ES to attenuate cytokine gene expression relative to IS was lost in two days and was now associated with markedly amplified and dysregulated cytokine gene expression. These data indicate that regulatory mechanisms exist in the brain that are responsive to stress and how the host perceives the stressful event.

3.5. Cytokine gene expression assessed by real-time PCR

We next sought to validate the cytokine gene expression profiles characterized by gene array analysis using real-time PCR assays. The choice of genes to interrogate was guided by the array data (see Fig. 3). Four patterns of gene expression emerged from this study. First, two cytokines (*Ccl2*, *Ccl5*) showed no statistically significant differences in gene expression for all treatment groups relative to NS/M. Second, ES but not IS or NS induced elevated

expression of cytokine mRNA (Ccl4). Third, ES and IS both enhanced gene expression (Ccl12, Cxcl10). Fourth, ES and IS both suppressed gene expression (osteopontin, OPN) relative mock-infected mice (NS/M) and ES was significantly suppressed relative to IS. Suppressed OPN levels could have influenced neuroinflammation and pathogenicity because VSV is a potent inducer of IFN-I. In an experimental autoimmune encephalitis model, IFN-I suppressed OPN levels in dendritic cells resulting in excessive Il-27 production, a dominant Th1 (not Th17) response that lead to a abnormal delayed disease phenotype (Shinohara et al., 2008). Whether OPN influenced a reciprocal relationship between Th17 and Th1 subsets in the VSV encephalitis model will require further study. It should be noted that the response of NS mice was not statistically significant from the mock-infected (NS/M) mice for all cytokine genes examined even though these mice were infected with VSV. Thus, both stress plus virus infection was required to detect a significant response relative to NS/M mice. These data support our contention that controllability differentially regulates the antiviral neuroimmune response.

3.6. Controllability impacts hippocampal neuroinflammatory responses

The disparate differences in the OB neuroimmune responses seen on days 1 and 3 PI may reflect the presence of replicating VSV on day 3 PI. To determine whether VSV titers influenced controllability outcomes, we characterized hippocampal gene expression profiles at day 3 PI. At this time point, VSV cannot be detected in the hippocampus by qPCR (data not shown). Multiple chemokine genes belonging to the CC (Fig.5a), CXC (c) chemokine families, CC receptors (b) as well as proinflammatory cytokines (d) were activated despite the physical absence of VSV to drive this response. Notably, expression levels were enhanced in mice subjected to IS; whereas, ES trained mice responded similarly to NS controls. Although inductions were not as robust as those seen in the OB (see B), expression of several genes reached statistical significance (Cxcl10, Il1-ra, Il15) supporting our view that controllability differentially regulates this hippocampal response as well. This pattern of gene expression is similar to the responses seen on day 1 PI in OBs. Expression profiles were regionally distinct. For example, CC chemokines Ccl2, 4, 5, and 7 as well as CXC chemokines Cxcl9, 10 and 13 were robustly induced in the OB but not in the hippocampus (B). Although this was generally true for most cytokine genes, exceptions existed such as Cxcl2 and Il10rb where inductions were robust in the hippocampus but not in the OB. Thus, stressor controllability differentially impacts the hippocampal antiviral immune response prior to pathogen arrival.

3.7. Effect of controllability on peripheral antiviral immune responses

We next examined whether the peripheral antiviral immune response was similarly regulated by stressor controllability. We characterized splenic cytokine profiles in the same mice that ere used to determine responses in the OB and hippocampus at day 3 PI. Similar to the CNS, multiple chemokines and proinflammatory cytokine genes were activated by VSV (Fig. 6A) likely reflecting the ability of the spleen to sequester this circulating viral pathogen and to respond immunologically. With the exception of Cxcr5, ES enhanced gene expression of CC (Ba), CXC (Bb) chemokines and proinflammatory cytokine genes (Bc) relative to IS or NS trained mice. Despite the generalized inflammatory environment, there was selective suppression of specific genes such as Cx3cl1 (fractalkine) and tollip (A) in the encephalitic

brain irrespective of the stressor. Gene expression profiles in the spleen were distinct relative to OB (C) as well as the hippocampus and amygdala (data not shown). Thus, controllability differentially regulates the peripheral and central antiviral immune response to VSV.

3.8. miRNA146a is a stress-responsive upstream regulator of the neuroimmune response to VSV

Because immune responses are driven by antigenic load, we tested whether the differential responses induced by controllability seen at day 1 PI reflected different viral loads. Fig. 7 demonstrates that OB VSV-N mRNA levels were similar in all cohort groups (A) indicating that viral antigen was not responsible for the amplified response seen in IS mice. This result suggested that ES and IS may trigger different signaling pathways in response to VSV neuroinvasion. Using the cytokine expression data from OB at day 1 PI, we employed Ingenuity Pathway Analysis (IPA) to determine what upstream regulation would be consistent with the observed pattern. IPA indicated only two microRNAs, miR-146a and miR155, as likely upstream regulators (URs) in our system at this time point. To test this prediction we examined miRNA146a levels in OBs of stress treated mice. We used IPA to determine a regulatory network from the >2-fold changed expressed genes and the two miRs (155 and 146a) that were indicated by IPA (B). Red in B means the indicated gene is more upregulated in IS versus ES, whereas, green means that gene is more highly expressed in ES compared to IS. Panel C shows a representative standard curve for the mir-146a loop PCR and panel D demonstrates that miRNA146a levels in OBs were significantly higher in mice trained with ES relative to IS mice. In more caudal brain regions, there was no significant differences in miR146a levels between ES and IS trained mice although significant differences existed between ES and NS cohorts (panels E, F). Thus, these data suggest that miR146a is a stress-responsive upstream regulator of the antiviral innate immune response and may play a key role in the differential responses induced by controllable and uncontrollable stress.

3.9. Controllability and chronic inflammation

The observation that mice subjected to controllable stress still displayed significant weight loss weeks after viral clearance suggests an ongoing process unrelated to the presence of VSV in the CNS. We therefore tested the hypothesis that stress, particularly controllable stress, facilitated a chronic neuroinflammatory response that contributed to persistent weight loss. Fig. 8 demonstrates (panels a-d) that mock-infected mice were essentially devoid of CD45^{high} inflammatory cells (a). A weak but visible infiltrate was present in NS mice (b). However, stress, especially in mice given ES, resulted in a more robust brain infiltrate than NS cohorts (c, d). The infiltrate contained a subset expressing CD103, a canonical marker for brain T resident memory (Trm) cells not expressed on peripheral T cells (e-h). Bevan's laboratory has characterized these cells and demonstrated that they remain activated and continue to express elevated levels of proinflammatory cytokine mRNA (Wakim et al., 2010; Wakim et al., 2012). Consistent with this view, we detected elevated expression of CD11b on CD103+ cells indicating an activated state because naïve T cells are CD11b- (i-k). We believe that this is the first demonstration that the behavioral control of stress impacts the development of a brain-adapted population of Trm cells that remain in an activated state long after VSV clearance from the CNS

4.0 Discussion

The current work demonstrates the complexity of the interaction between the stress and immune systems, and illustrates that the ability of an organism to engage in activities that can reduce aversive stimuli can have significance for immune activation and the ability to respond to pathogenic challenge. Differences in immune activation occur even though the paired ES and IS mice receive identical amounts of footshock and animals trained with ES and IS show similar activation of the stress system, as indicated by corticosterone (Maier et al., 1986) and SIH (Wellman et al., 2015; Yang et al., 2011). Thus, the ability of an animal to exert control on some aspects of the stressful situation and potential differences in the way stressors are perceived can have profound effects on stress-related outcomes.

We started training mice 3 days prior to VSV inoculation because learning the appropriate escape response (control) is critical to produce differences between ES and IS. In prior work, we demonstrated that only mice that showed improved escape latencies over time also showed increases in REM sleep (Machida et al., 2013). In this study, mice showed early improvements in escape latency prior to inoculation, thereby demonstrating they had learned the escape response; however, they also showed subsequent decrements in performance such that escape latency on day 6 of training was no better than that on day 1. These decrements may have been associated with the initial phases of sickness because a number of chemokines and proinflammatory cytokine mRNAs are readily detected in the brain as early as day 1 PI. For example, we have detected elevated brain mRNA levels for IL-1 α as well as IL1r1 one day PI (Steel et al., 2014). Some of these induced cytokines (IL-1, TNF) are known to cause sickness or cachexia, (Mutnal et al., 2011). The stress paradigm that we employed also allowed us to examine how stressor controllability impacted the induction phase (days 0-3 PI) of immunity, a time when numerous immunological events occur that are essential for innate and adaptive antiviral immunity

VSV is a relatively simple negative stranded RNA virus with a well-defined pathway of neuroinvasion into the OB and then into more caudal structures such as thalamus, hypothalamus, hippocampus, and pons as it travels caudally to the brainstem. VSV encephalitis has served as a prototypical model for acute virus infections of the brain. This neurotropic and cytopathic virus does not establish latency or persistence and is cleared completely from the CNS and periphery. Intranasal application of VSV initially results in infection and subsequent replication in olfactory receptor neurons with subsequent progression via the olfactory nerve into the OB. We previously reported that cytokine gene activation can be detected in the OB as early as 3 hrs PI and is robust by 12 hrs in unstressed mice (Ciavarra et al., 2017). Activation of these signaling pathways leads subsequently to a global bimodal chemokine and cytokine response with peak expression on days 3 and 7 reflecting the contribution of CNS resident and infiltrating T cells, respectively. As demonstrated here, chemokine genes were much more responsive to controllability than cytokines with the one exception being OPN. mRNA levels for this cytokine were suppressed by both ES and IS relative to shuttlebox exposed cohorts. Importantly, differential gene expression was evident for this cytokine; that is, ES significantly suppressed OPN expression levels relative to IS. OPN is known to function as a proinflammatory cytokine but its expression can lead to variable outcomes. For example,

OPN expression was associated with protection against West Nile Virus (WNV) encephalitis by controlling inflammasome components and neuronal apoptosis; whereas, in a second study OPN increased infiltration of WNV-infected neutrophils, viral burden and increased mortality via a “Trojan horse” mechanism (Ladwig et al., 2017; Paul et al., 2017). OPN is also thought to contribute to HIV-associated neurocognitive disorders because OPN expression is associated with elevated HIV replication in macrophages and enhanced cell-cell adhesion which can facilitate viral spread (Brown, 2015). Although the mechanism(s) by which OPN influences neuroinflammation in the VSV encephalitis model remains unclear, it is possible that ES-mediated suppression of OPN may have skewed microglia M1/M2 polarization to a more detrimental M1 phenotype (Ladwig et al., 2017) contributing to the enhanced morbidity and decrease survival seen in mice subjected to ES. As demonstrated in this study, OPN is just one of many chemokines and cytokines that were elaborated in the encephalitic brain necessitating additional studies to clarify its role in this model. In view of complex array of cytokines induced by neuroinvasive VSV, it is not surprising that one of the cardinal features of this model is the robust infiltrate of inflammatory cells that reaches its peak around one week PI. Neutrophils are the predominant inflammatory cell type but T cells and monocytes/macrophages are also well represented (Ireland and Reiss, 2006; Steel et al., 2014). At day 3 PI, this complex mixture of chemokines and proinflammatory cytokines reflects the contribution of CNS resident cells and not inflammatory cells because few inflammatory cells are present at this time (Steel et al., 2014).

We have extended these studies to determine whether controllability could differentially modulate pathogenesis and survivability. Importantly, controllability had a major impact on disease progression and survival. Mice subjected to ES displayed accelerated and sustained weight loss, a sensitive indicator VSV pathogenesis. Survival was also adversely effected by ES, but not by IS or simple exposure to the shuttle box. The observation that pathogenesis was greater in mice that experienced ES was somewhat surprising based the general conception that outcomes are generally better following controllable stress (Foa et al., 1992). The case for the detrimental effects of excess neuroinflammation has been amply made, e.g., its roles in diseases and disorders, like Alzheimer's disease, amyotrophic lateral sclerosis, epilepsy, Huntington's disease, multiple sclerosis and Parkinson's disease (Frankola et al., 2011; Frischer et al., 2009; Karagkouni et al., 2013), as well as neuropsychiatric disorders (Hagberg et al., 2012; Kato et al., 2011; Theoharides et al., 2014) and there obvious advantages to having a mechanism to keep stress-induced neuroinflammation in check. However, neuroinflammation also is an important process by which the brain responds to insults, and the initial immunosuppressing effect of ES likely interfered with the ability of the immune system to appropriately respond to VSV. The importance of immunosuppression may vary depending on whether the organism is challenged with a pathogen capable of replicating or a non-infectious agent as immunosuppression or excess anti-inflammatory cytokine concentrations could impair pathogen clearance mechanisms in the host (Opal and DePalo, 2000). Interesting, stress (inescapable tail shock) induced increased levels of the danger-associated molecular pattern (DAMP) protein HMGB1 (high mobility group box 1) and the NLRP3 inflammasome (Cheng et al., 2016). It is possible that the markedly different neuroinflammatory responses seen at day 3 PI reflects differential induction of HMGB1 by

ES and IS. It should also be noted that the differences between controllable and uncontrollable stress in mediating the effects of a subsequent challenge may also be impacted by other factors, such as stressor intensity and duration. For example, in rats, ES and IS produced similar potentiation of the microglia response in hippocampal tissue to lipopolysaccharide challenge in a training regimen of 100 tail shocks ranging from 1.3 to 1.6 mV in intensity (Frank et al., 2007). Thus, other factors that could significantly alter an animal's perception of the stressful situation may also mediate immune outcomes even if the stressor has some level of control.

Notably, weight loss in ES trained mice did not recover during the observation period perhaps suggesting an ongoing neuroinflammatory response. Consistent with this view, blood-derived CD45^{high} inflammatory cells remained especially in mice given ES. T cells represent one of the major infiltrating cell types during VSV encephalitis and become long-term brain Trm cells that potentially contribute to chronic changes in neuronal function as a result of persistent cytokine production (Wakim et al., 2010; Wakim et al., 2012). Consistent with these prior studies, activated (CD11b+) Trm cells were readily detected especially in ES trained mice at days 31 and 38 PI. We believe that this is the first demonstration that the behavioral control of stress impacts the development of a brain-adapted population of Trm cells that remain in an activated state long after VSV clearance from the CNS.

How stressor controllability impacted cytokine gene expression was dependent on the stage of virus progression. Initially, ES relative to IS and NS cohorts attenuated OB gene expression, a pattern that was reversed two days later. This initial amplified and dysregulated response may have contributed to an excessive neuroinflammatory response in ES trained mice that persisted, impacting pathogenicity/morbidity and Trm cell development. This time-dependent change may reflect different miRNA146a levels, an IPA predicted stress-responsive upstream regulator of this response. ES induced the highest levels of miR146a on day 1 PI and by day 3 miR146a levels were undetectable regardless of the treatment (data not presented). These data are consistent with prior work demonstrating a general role for microRNAs in regulating VSV replication in vivo and host survival (Otsuka et al., 2007) as well as a specific role for miR146a in regulating VSV replication in immune cells (Hou et al., 2009). However, INF β responses were strongly induced in mice treated with IS suggesting that IS activates another regulatory pathway. In this regard, VSV-induced miRNA155 may promote INF β production by suppressing SOCS1 (suppressor of cytokine signaling 1), a canonical negative regulator of the type I IFN signaling pathway (Wang et al., 2010). How stress-responsive miRNA146a and perhaps miRNA155 interact potentially to regulate neuroinflammation is currently being investigated.

Overall, these studies demonstrate that behavioral control of stress can be a major determinant of the quantitative and qualitative characteristics of two major innate antiviral signaling pathways in the CNS. Sensing of a distal viral pathogen by innate recognition/signaling pathways occurred early in the OB providing chemotactic gradients for blood-derived inflammatory cells coincident with viral pathogen arrival into the CNS. Stressor controllability had profound and varying effects across time on the central and peripheral immune response depending on viral progression. Initially in the OB (day 1 PI), IS promoted activation of Rig-I and type I IFN signaling pathways relative to ES and NS

cohorts that were independent of viral titer. In contrast, ES attenuated stress-induced amplification of these signaling pathways. However, the ability of ES to continue to mitigate cytokine gene activation was lost by day 3 PI in the OB but not the hippocampus. Indeed, cytokine responses were wildly amplified in ES mice and greatly exceeded responses in IS and NS mice. This suggests that ES and IS triggered different signaling pathways prior to VSV infection that impacted subsequent gene expression profiles. This view is consistent with preliminary Affymetrix gene array data demonstrating that just two training sessions with either ES or IS alone are sufficient to differentially activate 480 genes in the median prefrontal cortex (data not shown). It is likely that ES and IS also activate different genetic programs in other distinct brain regions and this may explain why ES continued to attenuate gene expression in the hippocampus, but amplified this response in the OB. Although the different signaling pathways activated by ES and IS across brain regions remain to be characterized, the differences likely involve different perceptual and emotional assessments of the stressor as well successful and unsuccessful escape learning. The importance of these factors on the neuroimmune response should not be underestimated because both pathogenicity and survivability were markedly impacted.

In conclusion, the current study demonstrates that whether or not a stressor is controllable can significantly impact the neuroimmune response with consequences for host defense and even survival. This appears to be related to the differential immunoactivating and immunosuppressive effects of uncontrollable and controllable stress. Thus, a clear understanding of the differential regulation of controllable and uncontrollable stress on immune function has important implications for improved insight into both the direct effects of stress on psychological health as well as its ability to modify responses to pathogens.

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Highlights

- Controllable and uncontrollable stress differentially impact viral pathogenicity.
- Controllable stress dysregulates cytokine responses following viral neuroinvasion.
- Controllable stress decreases survival during viral encephalitis.
- Differential stress-related cytokine responses are regulated via miR146a.
- Controllable stress increases persistent brain T resident memory cells.

Fig 1.

Behavior control of stress regulates pathogenicity and host survival to a neurotropic viral pathogen. (A) Escape latency across six days of training with ES and IS. These data demonstrate improvement in performance from day 1 of shock training through day 3 when VSV was administered (+, $p < 0.05$ day 3 compared to day 1). Post-infection, performance decreased (*, $p < 0.05$ day 6 compared to day 3). (B). Mice (6 mice/treatment group) were exposed to ES, IS or the shuttle box alone without foot shock (non-shock, NS) for 3 days and then given a single instillation of intranasal VSV. Training was terminated 3 days PI when some cohorts were euthanized for cytokine profiling (see Fig. 3). The remaining mice were weighed on the indicated days (B) and after 30 days survival calculated (C). Significant weight loss occurred for ES trained mice relative to either IS or NS cohorts (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). There was no significant difference in weight loss between IS and NS trained mice. Survival was significantly decreased in mice given ES relative to IS and NS cohorts (log-rank, Mantel-Cox Test, $p = 0.05$). No significant differences in survival were noted in mice given IS relative to NS mice. This experiment was repeated a second time (4 mice/treatment group) and yielded similar statistical results (data not presented).

Fig 2.

Early activation of olfactory bulb innate antiviral signaling pathways are amplified by uncontrollable stress. Mice (4-5/ group) were trained with ES or IS for 3 days, given intranasal VSV on the third day of training and then OBs evaluated one day PI for evidence of cytokine gene expression. A third cohort group was exposed to the shuttle box but not given foot shock and then infected with VSV (non-shock, NS). A fourth group was shuttle box exposed but given a single instillation of intranasal PBS (non-shock, mock infected, NS/M). (A) Scatter diagram illustrating major overall changes in gene expression relative to NS/M cohorts. Red lines represent >3 fold changes in gene expression. Bar graphs of major changes in gene expression in Rig-I(B) and type I IFN signaling pathways (C).

Fig 3.

Controllable but not uncontrollable stress profoundly amplifies and dysregulates cytokine gene expression following VSV neuroinvasion. Mice (4-5/group) were subjected to either ES or IS for 3 days and on the third day given a single instillation of intranasal VSV. Training continued for 3 additional days when mice were euthanized and OBs isolated for RNA profiling (A). Control mice were put into shuttle boxes but not given foot shock and then infected with VSV (non-shock, NS). OB RNA from each cohort group was pooled and cytokine profiles determined using a qPCR inflammatory cytokine array following the manufacturer's instruction (Qiagen, Catalogue No. PAMM-011D). Fold inductions were calculated relative to RNA levels detected in NS/M mice. In panels 8-G each panel is divided into thirds representing the response seen in mice given the indicated treatments. Data represent the mean $\pm$ SEM of two independent experiments.

Fig.4.

Cytokine gene array data assessed by individual real-time PCR. To verify cytokine gene expression, RNA was isolated from OBs obtained on day 3 P| and cDNAs generated from individual OBs using Qiagen's RT² First Strand Kit (Cat No./ID: 330404) following the manufacturer's instructions. mRNA levels for each sample were determined for the indicated cytokine and the house keeping gene HSP90. Data are ratios expressed as the average Ct value of triplicate determinations of house keeping gene hsp90/ gene of interest.

Fig. 5.

Hippocampus | cytokine responses are regionally distinct and remain attenuated by controllable stress. (A) Mice subjected to ES or IS training were given intranasal VSV and then euthanized to characterize hippocampal cytokine profiles at day 3 P.I. Data represent the mean $\pm$ SEM of two independent experiments (4-5 mice/group). Only responses that were >3 -fold (relative to non-shock, mock infected controls, NS/M, as defined in Fig. 2) are displayed for Cc chemokines (a), Cc receptors (b), Cxc chemokines (c) and cytokines and cytokine receptors (d). (B) Comparison of cytokine gene expression profiles for OB and hippocampus for Cc chemokines and receptors (e), Cxc chemokines (f), and cytokines and cytokine receptors (g) in NS mice given intranasal VSV. Changes in gene expression relative to NS/M mice.

Fig. 6. Peripheral antiviral immune responses are differentially regulated by stressor controllability. Splenic RNA was isolated from mice given either ES or IS and then VSV infected as described in Fig.2 legend. Control mice were exposed to the shuttle box, not foot shocked and then VSV infected (NS). Mock infected mice remained undisturbed in their cages but were given intranasal PBS. (A) Scatter diagram demonstrating overall splenic cytokine gene expression on day 3 PI in mice subjected to the indicated stressor. (B) Bar graph illustrating the major changes in gene expression in mice trained with the indicated stress paradigm. Values represent fold difference relative to undisturbed, mock-infected mice. (C) Bar graph illustrating distinct cytokine profiles detected in the central (OB) and peripheral (spleen) immune systems.

Fig 7.

miRNA146a levels in the encephalic brain are regulated by stressor controllability. Mice were subjected to either ES or IS for 3 days and infected with VSV on the last day of training. One day PI, mice were euthanized and the indicated brain regions isolated to obtain RNA. (A) OBs were assessed for VSV-N levels by qPCR. (panel B) Stress-responsive upstream regulation network predicted by IPA (Ingenuity Platform Analysis) involving genes from the antiviral innate immune qPCR array with expression changes consistent with miR-146a, miR-155 influence. (C) Standard curve plotting miRNA 146a copy number versus Ct values demonstrating sensitivity of Loop-primer TaqMan PCR assay. Impact of the indicated treatments on copy number of miRNA146a detected in OB (D), hippocampus (E) and PFC (F) at day 1 PI. Asterisks in panels D-F indicate significant differences between the indicated groups ($p < 0.05$).

Fig. 8.

Stressor controllability modulates development of Trm cells in the encephalitic brain. Mice were exposed to the ES, IS or NS conditions and infected intranasally with VSV. Control mice were NS/M. At 31 days PI whole brains were evaluated for Trm cells by flow cytometry. Dead cells were first excluded by forward and side scatter characteristics. Cells isolated from brains from VSV infected mice stained with isotype control monoclonal antibody (mAb) or the indicated mAbs. Microglia (G1, gate 1) and infiltrate (G2) were identified by CD45 and CD11b expression (a-d). Trm cells in the infiltrate (G2) were identified by CD103 expression (e-h). Back gating for CD103 expression in the G2 gate demonstrates the Trm cells are still activated (CD45^{high} CD11b⁺ i-k). This experiment was repeated a second time but harvested at day 38 PI; and yielded similar results.

Table 1

List of Primer Sequences Used for Real-Time PCR. The Gene Name, The GeneBank Accession Code, The Amplicon Size (In base pairs), The Sequence of the Forward (Fwd) and Reverse Primers are Indicated.

Gene	GeneBank	Amplicon Size	Sequence (5'-3')
Ccl2	NM_011333	368	Fwd TTCACCAGCAAGATGATCCCAATGAGT
			Rev ATATTAATTAAGGCATCACAGTCCGAGT
Ccl4	NM_013652	314	Fwd CTCTGTGCAAACCTAACCCGAGCAAC
			Rev GCTGCTCAGTTCAACTCCAAGTCACTCA
Ccl5	NM_013653	198	Fwd GAACCGCCAAGTGTGTGCCAACCCA
			Rev GATGCCCATTTTCCAGGACCGAGT
Ccl7	NM_013654	305	Fwd GCTATGTCAAGAAACAAAGATCCCC
			Rev CTATCCCTTAGGACCGTGATCAACA
Ccl12	NM_011331	172	Fwd ACATGAAGATTCCACACTTCTATGCCTC
			Rev TGGCTGCTTGTGATTCTCCTGTAGCTC
Ccr1	NM_009912	85	Fwd TTCAGATTTACAGAAGCCTACCCAC
			Rev GGCTCTTACAGCAGTCTTTTGCAAT
Cxcl10	NM_021274	119	Fwd ATGGTCCTGAGACAAAAGTAACTGCCGAA
			Rev CGCACCTCCACATAG CTTACAGTA CAGA
Il1b	NM_008361	226	Fwd TGAAGCAGCTATGGCAACTGTTCC
			Rev CTTCTCCACAGCCACAATGAGTGA
Spp1	NM_001204203	238	Fwd CAAGCAATTCCAATGAAAGCCATGACC
			Rev CTCAGTCCATAAGCCAAGCTATCACC

Additionally, RT² qPCR Primer Assays for Mouse Hsp90ab1 (NM_008302) were provided as a gift from Qiagen. The sequences for the Hsp90ab1 qPCR primer assays are proprietary and not provided by Qiagen.