Stress Regulation of Sustained Attention and the Cholinergic Attention System

Supplementary Information

Supplemental Methods

SAT shaping and behavioral measures

After behavioral shaping to train rats to nose poke for a food reward, rats were trained to discriminate between two types of trials: signaled (a signal light flashed at the top of the screen) vs. non-signaled (no light). Detection of the signaled trial was indicated by nose poking on the left response area on the screen, while detection of the non-signaled trial was indicated by nose poking on the right side of the screen (with locations for signaled and non-signaled trial responses counterbalanced between rats). Omissions were counted when rats did not poke either response area on a trial. In the initial training phases, signal durations were kept at 500ms until baseline criteria (>70% correct responses on signaled trials, >70% correct responses on non-signaled trials, and <20% omissions) was achieved for 3 consecutive days. Then rats were advanced to SAT.

For SAT, rats were trained to discriminate between signaled (durations 500ms, 50ms, or 25ms varied pseudorandomly) and non-signaled trials. They responded to each trial by touching specific response areas on the touchscreen to receive a food reward (45mg Precision Pellet, Bio-Serv). Omissions were counted when no response was made. All rats were trained on the SAT task until they reached passing criteria (>70% correct responses on 500ms signal trials, >70% correct responses on non-signal trials, and <20% omissions) for 3 consecutive days (for analysis these days were averaged to indicate baseline performance). Rats that did not make criteria were dropped as described(1). VS groups (male n=13; female n=10) were then exposed to daily stressors and

tested in SAT 30min after the cessation of each stressor. For the unstressed group (male n=16; female n=11), rats continued to run in SAT without any stressor exposure for 6 days after reaching passing criteria. All testing was done between 9am—5pm, with each rat tested 6 days/week around the same time.

Overall attentional performance was assessed with the vigilance index, based on the proportion of hits (h) and false alarms (f) as is standard with SAT. Vigilance index is calculated using the following formula: $Vigilance\ index = \frac{h-f}{2(h+f)-(h+f)^2}$ (2). The values of vigilance index range from −1 to +1 with a value of 0 indicating that the rat cannot distinguish between signaled and non-signaled trials and increasing positive values indicating increasing accuracy. Note that vigilance index does not include omitted trials, which were assessed separately.

Dendritic morphology

Initial anesthetization carried 4-5% Isoflurane via oxygen at a flow rate of 0.6ml/min. Isoflurane via oxygen flow at 0.6ml/min was maintained at 1-2% for the remainder of the surgery. The virus AAV9.CAG.Flex.eGFP.WPRE.bGH (provided by Dr. Hongkui Zeng of the Allen Institute for Brain Science and obtained through the University of Pennsylvania, Viral Vector Core) was bilaterally infused using a 10µL Hamilton syringe mounted to a Stoelting stereotaxic injector microinjection pump at 1µL, 1×10^9gc/µL per side in the nucleus basalis of Meynert (NBM). Injection coordinates used were as follows: -1.7mm posterior to Bregma, ±4.0mm lateral (male) or ±3.5mm lateral (female) to Bregma, and -7.5mm ventral from the surface of the skull. Rats in all surgical conditions received analgesic, Flunixin (Bimeda) 2.5 mg/kg, s.c., prior to and 24h after surgery. Rats were singly housed following surgery and allowed at least 2 weeks for full

viral expression before tissue collection. Half the rats underwent the VS procedure and half were controls.

Control and VS rats were deeply anesthetized with euthasol and were then perfused with 0.9% heparinized saline followed by 4% paraformaldehyde solution (for VS rats, perfusions took place 30 minutes after the cessation of the last stressor on day 6). Following transcranial dissection, brains were stored in a sucrose azide solution. Brains were cut into 200 μm coronal sections using the Vibratome 3000 Plus while submerged in ice-cold PBS. The sections containing NBM were mounted onto slides and cover slipped using prolong gold and kept from light exposure.

To validate that the virus was indeed labeling cholinergic neurons, we performed immunofluorescence using an anti-ChAT antibody (mouse anti-ChAT monoclonal antibody, Millipore, CAT: MAB305) at a 1:100 concentration for a 2-day incubation period using a protocol as previously described (3).

Sections were imaged with a Leica SPEII confocal coupled to a DMI4000 B microscope using a 25× water immersion objective (N.A.=0.95). Cholinergic neurons in the NBM region are relatively sparse, but have a large rostral-caudal extent(4, 5). Note that these neurons lack spines. When imaging, 2-3 cells with full dendritic trees distinguishable from neighboring cells were selected by a rater blind to the experimental condition from the rostral (-2.16mm and -2.64mm from Bregma) and middle (-1.72mm and -2.04mm) NBM, along with 1-2 cells from the caudal NBM (-2.20mm)(6).

Neurons were reconstructed and analyzed using Neurolucida (MBF Bioscience Inc., Williston, VT). The dendritic parameters analyzed included the number of nodes (i.e., branch points), the number of ends, and the total dendrite length. Sholl analysis was performed by placing

concentric circles radiating from the cell body in 20 μm increments and counting the number of dendrites that intersected with each circle and the length of dendrites between circles (6).

Amperometric recording electrode preparation

Ceramic-based platinum microelectrodes (Center for Microelectrode Technology) were coated with choline oxidase (EC Number 1.1.3.17; Sigma-Aldrich) and electropolymerized with meta-phenylenediamine (m-PD; Sigma-Aldrich) as described(7-9). Microelectrodes that exhibited a sensitivity of ≥3pA/μM and a limit of detection <400nM in *in vitro* calibration were used for *in vivo* recording sessions. All *in vitro* calibrations occurred on the day *in vivo* recordings were planned.

Amperometric recordings were conducted at 2Hz by applying a fixed potential of +0.7V and data was digitized with FAST-16 potentiostat (Quanteon). Background currents were stabilized for 60 min, following which brief pulses of potassium (70mM; 200nL) were locally applied to produce rapid depolarization of prefrontal cholinergic terminals. At the end of recording sessions, rats were perfused and brains were removed and processed for Nissl-staining to verify the placement of microelectrodes.

The amplitudes of choline signals, that reflect hydrolysis of newly-released ACh, were measured by change in currents on enzyme-coated channels from baseline current, and converted into μM equivalents of choline based on *in vitro* calibration. The averages of three signal responses/manipulation/animal were analyzed.

RNA extraction

The NBM was fully homogenized in 700ul Trizol with a pellet mixer (VWR, 47747-370) until no tissue clumps were observed. After incubated for additional 5 minutes at room temperature, 140ul chloroform was added to each tube, which was mixed vigorously for 1 minute. Sample was then centrifuged at 12000×*g* for 15 min at 4ºC. After centrifuge, the aqueous layer (~350ul) was removed from the sample and mixed with 600µl buffer RLT and 430µl 100% ethanol. Up to 700µl of the mixture was transferred to an RNeasy spin column placed in a 2ml collection tube, and was centrifuged for 15 seconds at 8000×*g*. The flow-through was discarded. This step was repeated with the remaining sample and the sample spin columns. Then 350ul buffer RW1 was added and centrifuged to discard flow-through. Next 80ul of DNAse I solution was added to the column and left at room temperature for 15 minutes. 700µl buffer RW1 and 500µl buffer RPE was then added to the spin column and centrifuged for 20 seconds at 8000×*g* to wash the membrane. RNeasy spin columns were placed in a new collection tube and centrifuged at full speed for 1 minute. RNeasy Mini spin columns were then transferred to a new tube, and RNA was eluted in 30µl H2O by centrifuging at 12000×*g* for 1 minute.

RNA and library prep for RNAseq

RNA from every NBM sample was extracted using RNAeasy micro kit using trizol, followed by DNAse I treatment, as described by the manufacturer (Qiagen). RNA integrity (RIN) and concentration were assessed using a bioanalyzer (Agilent). Libraries were constructed using the Nugen ovation library prep (NuGEN) without ribosomal RNA reduction starting with 200ng of RNA. Libraries were prepared using NuGen Ovation RNA-Seq System v2 from total RNA and sequenced by UCLA Neuroscience Genomic Center. Nugen Ovation library prep was chosen

because of our small amount of total RNA (150-300ng) and because this approach is tolerant of slightly degraded RNA. It uses a combination of oligo dT and random hexameter priming for 1st strand cDNA synthesis and no rRNA reduction so rRNA transcripts are present in the final library. Samples were barcoded for multiplexing and sequenced at 75 bp paired-end on Illumina HiSeq4000. Samples were pooled 16 per lane and sequenced at a depth of 130 million reads per sample. rRNA was then removed from fastq file using Bedtools intersectBed and rRNA annotation from Biomart. Alignment quality was examined using FastQC.

qPCR

cDNA synthesis was performed on total RNA using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). 1µg RNA, 500ng Oligo (dT), and 10mM each dNTP, were incubated for 5 minutes at 65°C and then chilled on ice for 2 minutes. 5×First Strand Buffer, 5mM DTT (final concentration), 40U RNaseOut, and 200 U Superscript III RT were then added. The 20µl reaction was incubated for 60min at 50°C followed by a final incubation at 70°C for 15 minutes for termination. The resulting cDNA product was quantified using Qubit to control for reverse-transcript efficiency and 50ng of product was used in each subsequent qPCR reaction. Quantitative PCR was carried out on a real-time detection instrument (ABI 7900HT Sequence Detection System) in 384-well optical plates using Sybr Green PCR Master Mix. One no template control was always included in each plate. The primers were designed to span exon boundaries. To validate the primers, the melting curved was examined using RNA extracted from wild-type animals prior to tests with VS and control samples. The primers are listed as follows: Dusp1_F ctgctttgatcaacgtctcg, Dusp1_R aagctgaagttgggggagat, Dusp10_F cgggcaccatgaaccaaaag, Dusp10_R gacctgagacaccgtcttgg, Rfc5_F ctgagtccagggcattcgtt,Rfc5_R cctctccagccactgaactg,

Klf4_F cccaccccatctgcagaaat, Klf4_R gtgggaaacttcggagcagt, Pdk4_F ctacgtagccagtgtttggga, and Pdk4_R cctgacctgcatcggacttt.

Supplemental Results

SI behavior results

For omissions, there was a day×condition interaction [$F(3.16, 145.42)=5.73$, $p=.001$, $\eta^2_{partial}= .111$], and Greenhouse-Geisser estimates of sphericity ($\varepsilon=.53$) were used because sphericity was violated ($\chi^2(20) = 184.2$, $p< .001$). To determine which group changed over time, repeated measures ANOVAs were conducted for control and VS groups separately. Omissions in control rats remained similar across days [$F(3.41, 88.7)=1.73$, $p=.161$], but changed in VS rats over time [$F(2.91, 61.71)=6.63$, $p=.001$, $\eta^2_{partial}= .232$], and sphericity was violated ($\chi^2(20)=101.57$, $p< .001$) so degrees of freedom were corrected ($\varepsilon=.49$). There were no effects of sex, sex×condition, nor sex×condition×day interactions for omissions.

The results that did not reach significance are reported as follows: For the vigilance index data at all stimulus durations combined, there were no effects of sex [$F(1,46)=1.25$, $p=.269$], nor sex × condition [$F(1,46)<1$], nor sex×condition×day interaction [$F(4.46,204.99)=1.55$, $p=.183$]. Similarly, no main effect of sex was found for any of the stimulus durations: 500ms [$F(1,46)<1$], 50ms [$F(1,46)=3.37$, $p=.073$], and 25ms [$F(1,46)=1.74$, $p=.193$]. Similarly, there were no sex×condition interactions or sex × condition × day interactions (respectively) for any stimulus duration: 500ms [$F(1,46)<1$], [$F(4.46,204.93)=1.40$, $p=.232$]; 50ms[$F(1,46)<1$], [$F(4.73,217.65)=1.08$, $p=.370$]; and 25ms [$F(1,52)<1$], [$F(4.60,211.80)=1.17$, $p=.324$]. For omissions, no effects of sex [$F(1,46)<1$], sex×condition [$F(1,46)<1$], nor sex×condition×day interactions [$F(3.16, 145.42)=1.37$, $p=.252$] were found.

SI morphology results

There was no effect of sex [$F(1, 96)=1.69$, $p=.197$, $\eta^2_{partial}= .017$] or interaction [$F(1, 96) < 1$, $\eta^2_{partial}=.001$] (Fig. 2a) for dendritic length. There was no effect of stress [$F(1, 96)< 1$, $\eta^2_{partial}= .001$], [$F(1, 96)<1$, $\eta^2_{partial}=.005$] nor an interaction [$F(1, 9=.189$, $p=.664$, $\eta^2_{partial}=.002$], [$F(1, 96)< 1$, $\eta^2_{partial}=.001$] for nodes and ends, respectively.

Supplemental Figures and Tables

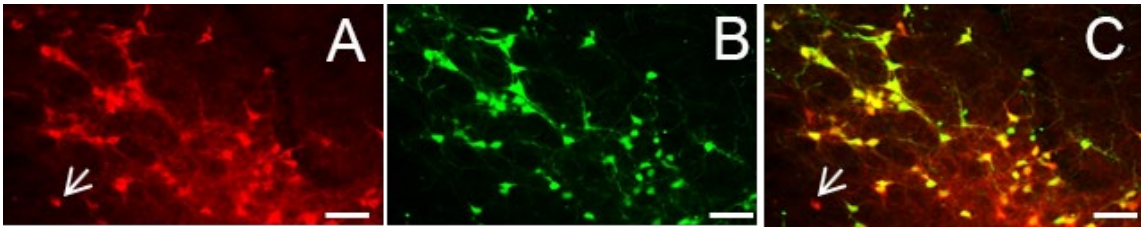

Figure S1. Technique to label cholinergic neurons. NBM cholinergic neurons in ChAT::Cre rats labeled with an anti-ChAT antibody in red (A), viral labeling of Cre-containing cells with eGFP in green (B), and the overlay (C). The arrow points to the only single-labeled cell.

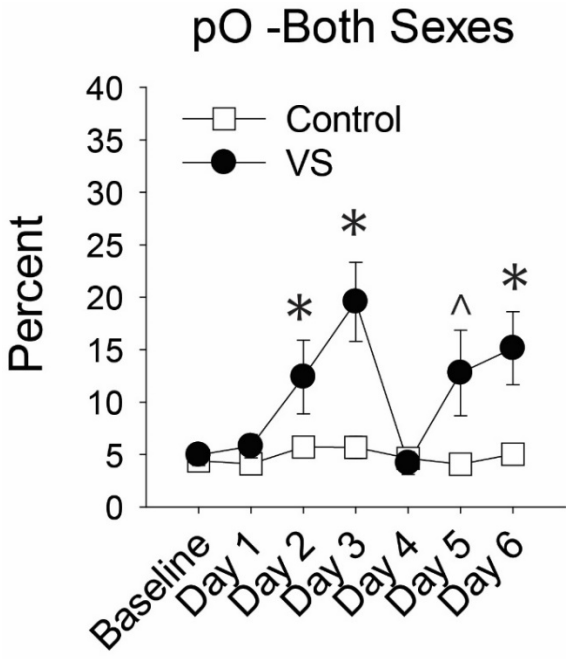

Figure S2. The percentage of omission (pO) changed in the VS group across training. Omissions increasing on stressor days 2, 3, 5, and 6. Asterisks indicate p< .05 from control group. Caret symbol indicates a trend, $p<.10$.

	KEGG Pathway	Number of genes	p-values
Male-specific	B cell receptor signaling pathway	4	5.01E-04
	T cell receptor signaling pathway	4	.002
	Hepatitis B	4	.004
	HTLV-I infection	5	.004
	cAMP signaling pathway	4	.009
	Pathways in cancer	5	.012
	Viral carcinogenesis	4	.016
Female-specific	Hepatitis B	4	.041
	Inflammatory bowel disease	3	.05

Table S1. KEGG pathway analysis on differentially expressed genes regulated by VS either in males (i.e., male-specific) or females (i.e., female-specific). VS increased the number of significant pathways more in males than in females. In both sexes, many pathways have to do with immune functions.

Supplemental References

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