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Stressful animal housing conditions and their potential effect on sympathetic neurotransmission in mice

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D'Arbe, M., R. Einstein, and N. A. Lavidis. Stressful animal housing conditions and their potential effect on sympathetic neurotransmission in mice. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R1422–R1428, 2002; 10.1152/ajpregu.00805.2000.—Although the sympathetic nervous system (SNS) plays a major role in mediating the peripheral stress response, due consideration is not usually given to the effects of prolonged stress on the SNS. The present study examined changes in neurotransmission in the SNS after exposure of mice (BALB/c) to stressful housing conditions. Focal extracellular recording of excitatory junction currents (EJCs) was used as a relative measure of neurotransmitter release from different regions of large surface areas of the mouse vas deferens. Mice were either group housed (control), isolation housed (social deprivation), group housed in a room containing rats (rat odor stress), or isolation housed in a room containing rats (concurrent stress). Social deprivation and concurrent stressors induced an increase of 30 and 335% in EJC amplitude, respectively. The success rate of recording EJCs from sets of varicosities in the concurrent stressor group was greater compared with all other groups. The present study has shown that some common animal housing conditions act as stressors and induce significant changes in sympathetic neurotransmission.

stressor; varicosities; social isolation; novel odor; vas deferens

VARIOUS RESEARCH GROUPS have studied ATP and norepinephrine (NE) release from sympathetic varicosities by using NE overflow (60), intracellular recording (10, 31), focal extracellular loose-patch recording (13, 14, 44), and amperometry (12, 53) techniques. Released ATP binds to postsynaptic $P2X_1$ receptor ligand-gated ion channels that allow cations to move into the postsynaptic muscle cell, causing depolarization (11, 13, 16, 48), whereas NE binds to α_1-adrenoceptors that activate a metabotropic contraction (15). The current generated when ATP binds to purinoceptors has been recorded with loose-patch electrodes and is known as the excitatory junction current (EJC; Refs. 13, 14). Because not every stimulus evokes an EJC, neurotransmitter release is said to be intermittent (13, 14, 44). Studies have indicated that the probability of neurotransmitter release is highly nonuniform, not only between sympathetic varicosities within a preparation but also between preparations and species (e.g., mouse vs. guinea pig; Refs. 13, 14, 19, 44).

Stress has been defined as a state of threatened homeostasis (58a, 61) and can be caused by physical (inflammation, hemorrhage, immune challenge) or psychological (excessive noise and light, overcrowding, social and maternal deprivation, handling, unfamiliar procedures) stressors. Exposure to stressors can be acute or chronic, with each producing different effects on the body (2, 3, 9, 22, 23, 34, 36, 39, 40, 54, 58). Because the sympathetic nervous system mediates many of the peripheral actions of the stress response (51), environmental conditions that cause stress may also affect the variability in probability of neurotransmitter release between varicosities of the sympathetic neuroeffector junction. An increase in the variance of the probability of transmitter release occurs after chronic exposure of animals to handling and minor surgical procedures (20). Other environmental stressors, including the housing conditions of animals, transportation of animals, and cagemate interactions, may also affect sympathetic neurotransmission. Two of the most common potential animal housing stressors are social deprivation and predatory threat (17, 49). The aim of this study, therefore, was to evaluate the effects of social deprivation and rat presence (as the predatory threat) on the probability of neurotransmitter release from sympathetic varicosities of the mouse vas deferens.

METHODS

Animals. Inbred male mice (BALB/c) aged 6 wk postnatal (Central Animal Breeding House, Pinjarra Hills, Queensland, Australia) were randomly separated into four groups. Each group of animals was housed in the conditions described below. Control mice (*group 1*) were group housed, whereas mice in the social isolation group (*group 2*) were singly housed. Mice in the rat odor group (*group 3*) were group housed, whereas mice in the concurrent stressor group

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(*group 4*) were singly housed in a room containing rats. All groups were housed for 7–14 days before being used for an experiment, with mice in *groups 1* and *2* housed for all days in the range. Mice in *group 3* were housed for 7, 8, 9, and 11 days in their respective housing conditions, whereas mice in the concurrent stressor group were housed in conditions for 7 days. Group-housed mice (*groups 1* and *3*) were maintained in white, opaque cages measuring 48.0 × 18.6 × 13.0 cm [length (L) × width (W) × height (H)], whereas socially deprived mice (*groups 2* and *4*) were maintained in white, opaque cages measuring 31.0 × 13.0 × 12.0 cm (L × W × H). Group-housed mice were housed with a minimum of two and maximum of four animals per cage. The number of mice per cage was kept constant for all experiments. Mice in *groups 3* and *4* (rat odor exposure groups) were housed in a room that also contained rats. Rat-containing cages were between 30 and 100 cm from mice-containing cages, so that only olfactory and auditory stimulus cues were detectable by the mice. Food and water were available ad libitum. All animals were kept in rooms with a 12:12-h light-dark cycle, a temperature of 20–22°C, and a background noise level of <60 dB. All animals were handled once a week for bedding changes.

Transportation of animals. Before each experiment, a mouse was selected from the appropriate cage, carefully removed, and placed in a transport cage. The transport cage measured 31.0 × 13.0 × 12.0 cm (L × W × H), with clean bedding, and was covered with paper towel. The cage was then carried from the animal house to the laboratory (a distance of ~20 m), and the mice were killed immediately upon arrival at the laboratory. All mice were transported in this way to minimize any variability resulting from this process.

Preparation of tissues. Animals were killed by cervical fracture. Both vasa deferentia were dissected from the animal, stripped of their connective and adipose tissue, and placed in a 3-ml bath, pinned to a bed of Sylgard (Dow Corning). Preparations were continuously perfused with Tyrode solution (in mM: 123.4 NaCl, 4.7 KCl, 1.0 $MgCl_2$, 1.3 NaH_2PO_4, 16.3 $NaHCO_3$, 2 $CaCl_2$, and 7.8 glucose) at a rate of 3 ml/min. The solution in the reservoir supplying the bath was continuously gassed with 95% O_2-5% CO_2, and the pH was maintained at 7.4. The temperature of the bathing solution was kept between 32 and 34°C. The mouse vas deferens was chosen for this study because of its importance over the last three decades as a model for sympathetic neurotransmission (5–8).

Stimulation of tissue. The prostatic end of the vas deferens was gently drawn into a glass pipette filled with Tyrode solution. Two Ag-AgCl wires, one on the outside and one on the inside of the glass pipette, acted as the stimulating electrode. The vas deferens was stimulated (Grass SD9 stimulator coupled to a World Precision Instruments A360 isolator) with trains of square-wave pulses of 0.08-ms duration and 15- to 20-V intensity applied at 0.16 Hz.

Visualization of tissue. The bath was placed on a microscope stage (Olympus BH-2), and the preparations were viewed using ×10 or ×20 objectives. A charge-coupled device (CCD) camera (Panasonic WV-BP310/G or WV-1900/B) was used to view the tissue on a monitor (Panasonic WV-BM 1400). The location of the recording area on the vas deferens was between 2 and 4 mm from where the vas deferens entered the stimulating electrode. A grid formation of 3 × 3 squares, with each square measuring 25 × 25 mm, was drawn on an overhead transparency sheet. This sheet was placed on the monitor, and important structures such as the edges of the vas deferens and the outline of the lumen were traced onto the sheet. Each 25 × 25 mm square was equivalent to 110 × 110 μm of vas deferens area when the ×10 objective was used or 55 × 55 μm of vas deferens area when the ×20 objective was used. Once the recording electrode and the tissue were both in focus and a suitable area of the vas deferens had been located, recording commenced.

Recording. An extracellular recording electrode (10- to 20-μm tip diameter) filled with Tyrode solution was placed on the surface of the vas deferens to record the nerve terminal impulse (NTI) and EJCs. The recording electrode was placed in the corner regions of each square when the ×10 objective was used so that up to 36 recording sites were obtained (4 positions in each square). When the ×20 objective was used, the electrode was placed in the left, center, and right regions of each square (3 positions in each square), so that up to 27 recording sites were obtained. The large number of recording sites ensured a large sample number for each group. If no EJCs were observed in the first 20 stimuli at any electrode position, it was assumed that there was no neurotransmitter release occurring at that electrode position, and the electrode was moved to the next location. If one or more EJCs were observed during the initial 20 stimuli, the responses to a total of 75 stimuli were recorded. This procedure was repeated for each electrode placement.

Data acquisition and analysis. For each site, data were digitized using a MacLab/2e (ADInstruments) and stored using Scope (version 3.3.3) software on a Macintosh computer (7200/120). The amplitudes and rise times of EJCs were measured using Translate (version 3.1) and Igor (version 2.02) software.

Because all EJC amplitude distributions were highly positively skewed, median EJC amplitudes and EJC success rates were compared between groups using the Kruskal-Wallis (KW) ANOVA test (nonparametric) with InStat software (GraphPad, version 2.03). Specific groups were compared using an a priori Dunn's multiple comparisons test. A result was considered significant if $P \leq 0.05$. Because the KW ANOVA test is nonparametric, the results will emphasize differences between group medians, rather than group means. However, the mean ± SD is given in brackets after a median group value.

Comparisons of EJC amplitude and EJC success rate to determine the presence of adaptation for the social deprivation group were done using a parametric Student's *t*-test, unless the assumption of equal standard deviations for the two groups was not met, in which case a nonparametric Mann-Whitney *t*-test was done.

RESULTS

Extracellular recording. The recording of EJCs with focally positioned extracellular electrodes served as indicators of neurotransmitter release (ATP; Ref. 32). Ten traces recorded from a vas deferens from one control mouse (Fig. 1*A*) and ten traces recorded from a vas deferens from one concurrent stressor mouse (Fig. 1*B*) are shown, with the stimulus artefact (SA), NTI, and EJC indicated. Three important features can be seen in this set of traces: the consistent NTI, the intermittent nature of recording EJCs, and the variable amplitude of EJCs. This shows that the intermittence in neurotransmitter release is not caused by action potential propagation failure but by a failure in the depolarization-secretion coupling mechanism.

To more accurately estimate the level of neurotransmitter release from a preparation, it was necessary to record from a large area of the vasa deferentia. The use

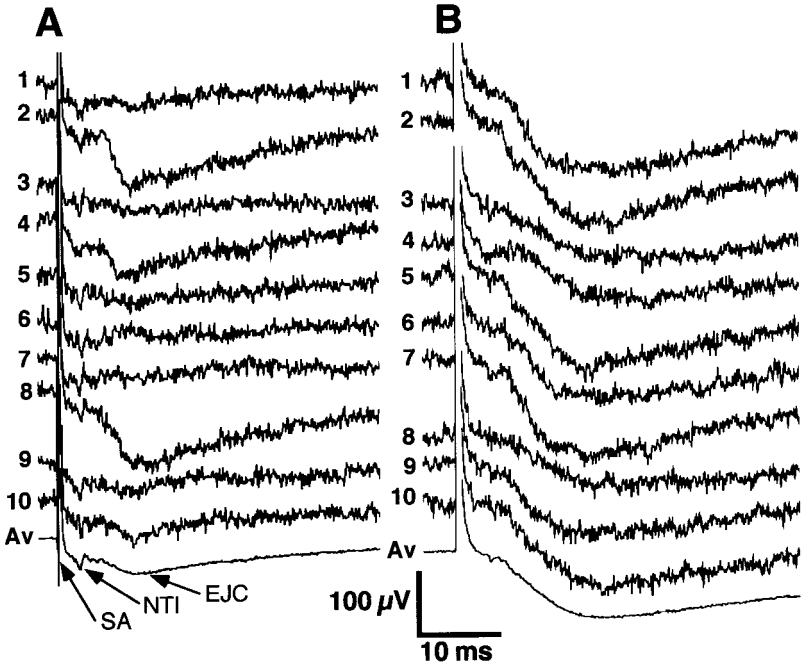

Fig. 1. Extracellular recordings of neurotransmitter release from a set of varicosities from a vas deferens from 1 control group mouse (*A*) and from a set of varicosities from a vas deferens from 1 concurrent stressor group mouse (*B*). Representative sets of 10 consecutive traces (*1–10*) are shown for each set. Stimulus artefact (SA), nerve terminal impulse (NTI), and excitatory junction current (EJC) are indicated on the average (Av) trace of *set A* (trace Av). The average from each set of 75 traces recorded by the 2 electrode placements is shown at the bottom of the 10 traces shown. The recording electrode tip diameter was 10–20 μm. Sympathetic axons of mouse vasa deferentia were stimulated with square wave pulses of 15-V intensity and 0.08-ms duration at 0.16 Hz.

of a grid system reduced the bias of selecting recording sites with any particular neurotransmitter release characteristics. Neurotransmitter release at each electrode placement was measured by using two parameters: the success rate of recording EJCs from a site and the EJC amplitude. An example of the distribution of EJC amplitude and EJC success rate for a set of recordings over the surface of a vas deferens from a single control animal and a set of recordings from the concurrent stressor animal is shown in Fig. 2. Figure 2, *A* and *C*, shows the distribution of mean EJC amplitudes in a vas deferens from a control mouse (Fig. 2*A*) and from a concurrent stressor mouse (Fig. 2*C*). EJC amplitudes recorded from concurrent stressor mice were consistently higher than those recorded from control mice. Figure 2, *B* and *D*, shows the distribution of EJC success rates of the same sites as shown in Fig. 2, *A* and *C*, respectively. The EJC success rates recorded from the concurrent stressor mouse vas deferens (Fig. 2*D*) were greater than those recorded from the control mouse vas deferens (Fig. 2*B*). Changes in the success rate of recording EJCs was used as an indicator of presynaptic changes, whereas changes in EJC amplitude may reflect pre- or postsynaptic changes.

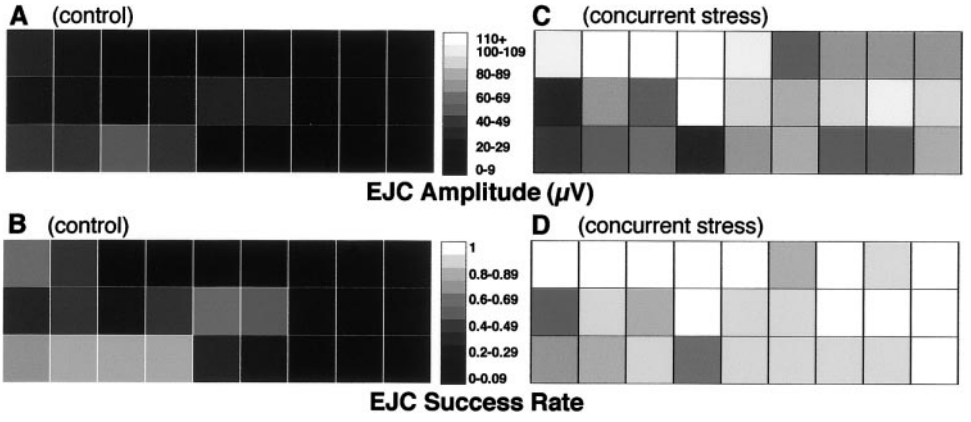

Fig. 2. Spatial distribution of EJCs over the surface of a vas deferens from 1 control mouse and the spatial distribution of EJCs over the surface of a vas deferens from 1 concurrent stressor mouse. *A* and *C*: distribution of average EJC amplitudes on the surface of the vas deferens for control mouse (*A*) and concurrent stressor mouse (*C*) when the recording electrode is placed in the positions indicated by each square. At each electrode placement, the responses to a total of 75 stimuli were recorded. The darkness (large EJC amplitudes shown by lighter shades of gray) of the rectangles indicates the range within which the average EJC amplitude for that electrode placement lies, as indicated by the legend. *B* and *D*: distribution of EJC success rates over the surface of a vas deferens for control mouse (*B*) and concurrent stressor mouse (*D*) when the recording electrode is placed in the positions indicated by each rectangle. At each electrode placement, the responses to a total of 75 stimuli were recorded. The darkness (large EJC success rates shown by lighter shades of gray) of the rectangles indicates the range within which the obtained EJC success rate lies, as indicated by the legend. EJC amplitudes and EJC success rates were calculated from the same electrode placements. The area of vas deferens surface that each grid represents is 165 × 165 μm.

Effects of stress on EJC amplitude. The effects of stressors on sympathetic neurotransmission were examined by measuring EJC amplitudes, the results of which are summarized in Fig. 3. There was a significant difference in the median EJC amplitudes using the KW ANOVA test between the four treatment groups (KW = 100.46; $P < 0.0001$; Fig. 3). A Dunn's multiple comparison a priori posttest revealed that the median EJC amplitude was significantly larger (30%; $P < 0.05$) in vasa deferentia from socially deprived mice (316 recording sites from 12 vasa deferentia) than control mice (306 recording sites from 15 vasa deferentia). Control mice had a significantly ($P < 0.05$) lower median EJC amplitude [23 μV (31 ± 28 μV; mean ± SD)] than the socially deprived group [30 μV (39 ± 35 μV)]. The median EJC amplitude was not significantly affected by rat odor. The median EJC amplitude from vasa deferentia from mice in the concurrent stressor group (105 recording sites from 5 vasa deferentia) of 77 μV (74 ± 37 μV) was significantly larger than that of all other groups ($P < 0.001$ for all comparisons; Fig. 3). The concurrent stressor group had a median EJC amplitude 335% greater than that of the control group, 296% greater than that of the socially deprived group, and 257% greater than that of the predator stress group (142 recording sites from 9 vasa deferentia). There was no difference in EJC amplitude between stressor exposure durations. Mean EJC amplitude for exposures of <9 days was 41 ± 2.6 μV (mean ± SE) compared with a mean EJC amplitude of 37 ± 2.9 μV for exposures of >9 days [$t_{314} = 1.0154$; $n = 316$, P = not significant (NS)].

Effects of stress on EJC success rate. Because neurotransmitter release from sympathetic varicosities is intermittent, not all stimuli result in EJCs. The success rate of recording EJCs for electrode placements that registered neurotransmitter release in the first 20 stimuli was calculated and is shown in Fig. 4. The success rate of recording an EJC was significantly higher in vasa deferentia from stressed animals than those from animals in the control group (Fig. 4), as shown by a KW nonparametric unpaired ANOVA (KW = 103.00; $P < 0.0001$). A Dunn's multiple comparisons a priori posttest showed that the median EJC success rate was significantly higher in the concurrent stressor group (105 recording sites from 5 vasa deferentia) compared with all other groups ($P < 0.001$ for all comparisons). The median EJC success rate in the concurrent stressor group [0.97 (0.84 ± 0.25)] was 236% greater than the control group [0.41 (0.47 ± 0.32)], 171% greater than the socially deprived group [0.56 (0.54 ± 0.31); 316 recording sites from 12 vasa deferentia], and 209% greater than the rat odor group [0.46 (0.50 ± 0.34); 142 recording sites from 9 vasa deferentia]. Although not statistically significant, the EJC success rate in the socially deprived group was 37% larger than the control group, whereas the predator stress group had an EJC success rate 13% larger than the control group. The success rate was not affected by the duration of stressor exposure. The average EJC success rate for social deprivation of <9 days was 0.57 ± 0.0227 (mean ± SE), whereas the mean EJC success rate was 0.51 ± 0.0282. The success rates were not significantly different when compared by using a nonparametric Mann-Whitney U t-test (U = 11,180, $n = 316$; NS).

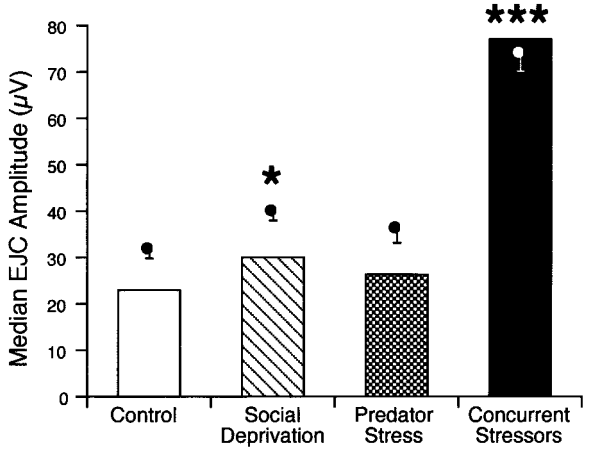

Fig. 3. Graph shows the effect of stressor exposure on EJC amplitudes for all 4 groups. Bars indicate the median EJC amplitudes for the groups. Dots associated with each bar represent the means of the groups, with the line underneath each dot indicating the SE. For clarity, only the negative part of the SE is shown. Statistically significant differences between treatment groups and the control group only are indicated (*$P < 0.05$, ***$P < 0.001$).

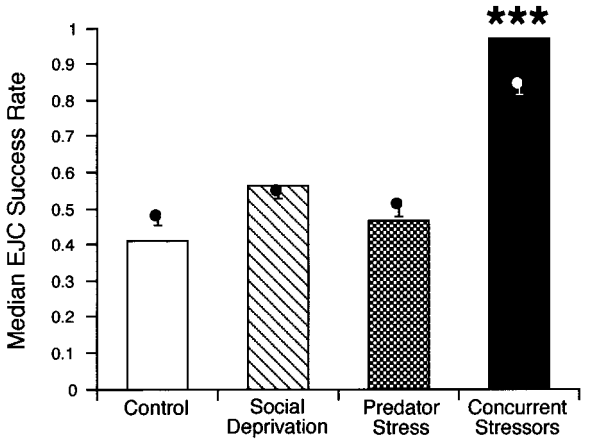

Fig. 4. Graph shows the effect of stressor exposure on EJC success rates for all 4 groups. Bars indicate the median EJC success rate for the groups. Dots associated with the bars represent the mean EJC success rates of the groups, with the line underneath each dot indicating the SE. For clarity, only the negative part of the SE is shown. Statistically significant differences between treatment groups and the control group only are indicated (***$P < 0.001$).

Effects of stress on the success rate of obtaining neurotransmitter release from a given electrode placement. The electrode was placed in a series of positions, which were predetermined by a grid (Fig. 2). Some electrode placements did not record an EJC within the first 20 consecutive stimuli (0.16 Hz) and thus were noted as failures in eliciting an EJC. This section examines the effect of stress on the success rate of an electrode placement to yield neurotransmitter release. The success rate to record neurotransmitter release on a given

electrode placement was compared across each of the groups. A parametric ANOVA showed no statistically significant differences between the groups. Although the differences between the groups failed to reach statistical significance, vasa deferentia from the concurrent stressor group showed that every electrode placement was successful at recording EJCs (105 recording sites from 5 vasa deferentia) compared with 77.7 ± 5.7% (mean ± SE) for control vasa deferentia (306 recording sites from 15 vasa deferentia), 75.4 ± 4.4% for the social deprivation group (316 recording sites from 12 vasa deferentia), and 68.5 ± 10.2% for the rat odor group (142 recording sites from 9 vasa deferentia).

DISCUSSION

Considerable nonuniformity exists in the level of intermittence between sympathetic varicosities, not only within a preparation, but also between different preparations from the same (44, 45, 59) and different animal species (19). For example, the average probability of neurotransmitter release from varicosities innervating blood vessels varies between 0.002 and 0.02 (4). The average probability of neurotransmitter release from varicosities innervating the guinea pig vas deferens varies from 0.005 to 0.03 (13, 14). Varicosities innervating the mouse vas deferens have an average neurotransmitter release probability of between <0.05 and 0.44 (42, 43). Although the differences in neurotransmitter release probability may be related to species variation, due consideration has not been given to the effects of exposure of animals to stressful conditions encountered before the experiment. The present study investigated whether common animal housing conditions affect the levels of sympathetic neurotransmitter release.

The most common animal housing conditions encountered by experimental animals include social deprivation and exposure to novel odors. The present experiments showed that there was a significant increase in neurotransmitter release after 7–14 days of social deprivation. There was an even greater increase in neurotransmitter release when animals were concurrently exposed to social deprivation and rat odor. The increase in neurotransmitter release was indicated by increases in EJC amplitude and EJC success rate (decreased intermittence). These results are consistent with a previous study showing an increase in sympathetic neurotransmission after exposure to the chronic stress of handling and saline injections (20). The present study has demonstrated that other, commonly encountered stressors, such as social deprivation and rat odor exposure, can also increase the probability of neurotransmitter release from sympathetic varicosities and that when two stressors are present at the same time, the effect is greatly increased. The present results showing that sympathetic neurotransmission can be significantly modified by common animal housing procedures highlight the need for better management of animal housing conditions and the need to reduce exposure to stressors.

The mouse vas deferens responds to α_2-adrenoceptor and opioid receptor-mediated chronic presynaptic inhibition of neurotransmitter release by an adaptive increase in neurotransmitter release (24, 25, 42, 46). The increase in neurotransmitter release observed in the present experiments, although smaller than those observed in other studies (24, 25, 42, 46), may reflect an underlying adaptation of sympathetic varicosities to increased circulating α_2-adrenoceptor and opioid receptor agonists during stressor exposure. Levels of epinephrine and NE (both α_2-adrenoceptor agonists) and endogenous opioids (for example, met-enkephalin, leu-enkephalin, and endorphin) are increased during chronic exposure to stressors (28, 39, 40, 51, 55). Rat tail arteries are less responsive to opioid agonists after chronic social deprivation (55), and this tolerance may be in response to increased levels of endogenous opioid and adrenergic mediators circulating in the stressed animal.

Chronic morphine treatment (CMT) leads to adaptive changes, both structural (37) and functional (24, 25). Stressor exposure increases opioid (28, 52, 55) and catecholamine (39, 40, 51) levels, which may produce adaptive changes similar to those observed after CMT (25, 42). These changes require at least 6 days to occur and persist after the tissue has been isolated. Adaptive changes are therefore unlikely to be apparent after acute stressor exposure. It is possible that the extent of adaptive change at the cellular level may depend on the animal's response to the stressor over time. Decreases in catecholamine (39) and endogenous opioid (34) levels as a result of stressor adaptation or habituation may reduce the development of adaptive changes in the vas deferens. On removal of the vas deferens from the in vivo environment, no sympathetic neurotransmitter release increase would be observed, and neurotransmitter release levels would be similar to control housed animals. The adaptive changes could include one or a combination of the following: an increase in Ca^{2+} channel density, an increased expression of vesicular associated proteins, or an increase in vesicle density and availability.

The present experiments show that exposure to stressors increases sympathetic neurotransmitter release levels. The greatest increase in EJC amplitude and EJC success rate occurred when mice were simultaneously exposed to social deprivation stress and novel odor stress, perhaps because this particular stressor combination elicits a greater stress response. Because stress has a significant effect on neurotransmitter release, inadvertent stressor exposure needs to be minimized in experimental animals. This is important, not only to satisfy animal welfare objectives but also so that the variability in the probability of neurotransmitter release between varicosities can be reduced and experimental data are not confounded by unnecessary negative influences.

Perspectives

The sympathetic nervous system regulates the reproductive system, cardiovascular system, gastrointestinal system, and immune system (21, 38, 41, 50). Thus any change in sympathetic nervous system function is going to have widespread effects on the organism. Two of the most intensively studied areas in the stress field are the relationships between stress and hypertension and between stress and immune function.

There is extensive literature that examines the effects of catecholamines on immune function. Electrical stimulation of regional sympathetic nerves results in an increased lymphatic pumping, which then affects lymphocyte output (26). In addition, peripheral stimulation of sympathetic ganglia produces suppression of T-cell proliferation, natural killer cytotoxic responses, and interleukin-2 and interferon-γ production (27, 32, 33), with these effects being blocked by chemical sympathectomy or administration of β-adrenoceptor antagonists (33). The present study may, therefore, have important implications for how housing conditions might affect immune function through sympathetic innervation of lymphoid organs. Comprehensive reviews on the role of the autonomic nervous system in immune function are available (26, 29).

This study may also have implications for how housing conditions may change the function of sympathetic axons innervating blood vessels and the subsequent effect on blood pressure. The effect of any adaptive changes that may be occurring in the varicosities innervating the blood vessels (56) could be important in the increase in blood pressure that has been observed after stressor exposure in several studies (1, 30, 35, 47, 57).

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