Sex-, stress-, and sympathetic post-ganglionic-dependent
changes in identity and proportions of immune cells in the dura

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Abstract

**Aim of investigation**—Due to compelling evidence in support of links between sex, stress, sympathetic post-ganglionic innervation, dural immune cells, and migraine, our aim was to characterize the impacts of these factors on the type and proportion of immune cells in the dura.

**Methods**—Dural immune cells were obtained from naïve or stressed adult male and female Sprague Dawley rats for flow cytometry. Rats with surgical denervation of sympathetic post-ganglionic neurons of the dura were also studied.

**Results**—Immune cells comprise ~17% of all cells in the dura. These included: macrophages/granulocytes (“Macs”; 63.2% of immune cells), dendritic cells (0.88%), T-cells (4.51%), natural killer T-cells (0.51%), natural killer cells (3.08%), and B-cells (20.0%). There were significantly more Macs and fewer B- and natural killer T-cells in the dura of females compared with males. Macs and dendritic cells were significantly increased by stress in males, but not females. In contrast, T-cells were significantly increased in females with a 24-hour delay following stress. Lastly, Macs, dendritic cells, and T-cells were significantly higher in sympathectomized-naïve males, but not females.

**Conclusions**—It may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women.

**Keywords**

Headache; meninges; inflammation; autonomic

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Introduction

Evidence suggests that a “sterile” inflammation of the dura is responsible for the activation and sensitization of dural afferents, which is thought to be responsible for migraine pain (1–3). Dural mast cells (4) and macrophages (5) may play critical roles in this process. While the extent to which other immune cell types may contribute to migraine remains to be determined, the clinical manifestations of migraine suggest several additional reasons to focus on dural immune cells as underlying contributors to this neurological disorder. First, the prevalence of migraine is approximately three-times higher in women than in men (6), and there are sex differences in immune cell number, phenotype, and regulation (7–10). Second, approximately 80% of migraineurs report stress as a trigger for a migraine attack and 60% report it as their main trigger (11), with attacks often occurring during the relaxation phase after stress (“stress–relaxation” model) (12). That time-dependent changes in the regulation of immune cells could account for the delay in the migraine attack after the resolution of the stressor is suggested by the observation that there are time-dependent shifts (13,14) and sexually dimorphic expression (15–18) of adrenergic receptors (ARs) on immune cells, which regulate pro- versus anti-inflammatory cytokine production (14,19). Third, there is evidence of sympathetic dysregulation in migraineurs (20) and evidence that α-AR agonists and β-AR antagonists can decrease the frequency of migraine attacks (21). Finally, the link between these clinical features of migraine and immune cells is suggested by observations that stress is associated with the activation of both the hypothalamic–pituitary–adrenal and the sympatho–adrenal axes, the dura is heavily innervated by sympathetic post-ganglionic neurons (SPGNs) (22,23), and immune cells are under AR regulation.

Despite this compelling evidence in support of a link between dural immune cells and migraine, there have been no systematic analyses of immune cells in the dura, let alone studies to address sex differences and/or stress-induced changes in these immune cells that would be consistent with a role in migraine. In order to begin to address this issue, we characterized the immune cells in the dura of male and female rats, as well as the impact of stress and SPGN innervation on the proportion of different immune cell types present. Our prediction was that stress would drive delayed SPGN-dependent changes in immune cell subtypes in females that would be consistent with the penultimate step prior to the initiation of an attack in migraineurs.

Methods

Animals

Pathogen-free adult (P50–70) male (200–300 g) and female (150–250 g) Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) were used for all experiments. All procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and performed in accordance with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Details of animal care and methods of the chronic variable stress (CVS) paradigm, surgical sympathectomy (SCGx), analysis of plasma corticosterone (CORT), isolation of cells from
the dura, flow cytometry, fluorescence-activated cell sorting (FACS), cell histology, and immunohistochemistry (IHC) are provided in supplemental text.

**Chronic variable stress**

Animals were exposed to CVS, consisting of an ongoing series of “mild” stressors applied for variable durations over a period of 4 days (Supplemental Figure 1). Stressors included: food or water deprivation, overnight illumination, cage tilt, paired housing with a stranger, damp bedding, white noise, strobe light, and predator odor (supplemental text).

It is important to note we are not proposing that CVS is a model of migraine, nor do we anticipate that this stress paradigm causes migraine in rats. Rather, we have proposed to study changes in response to stress because we hypothesize that these changes set the stage for the initiation of an attack in migraineurs.

**Data analysis**

Four related *a priori* hypotheses based on the clinical features of migraine were tested. First, there are more immune cells in the dura of females than males. Second, stress increases the relative proportion of dural immune cells with a delay following the cessation of stress that is greater in females than males. Thus, we examined the influence of both sex and stress on the proportion of immune cell subtypes in the dura by comparing naïve rats with rats exposed to 4 days of CVS, assessed either immediately following stress (CVS+0 h) or with a 24-hour delay following stress (CVS+24 h) (Figure 1a). The choice of a 24-hour delay was based on results from preliminary experiments also including a 12-hour delay group (n=4 for both males and females). No significant differences were detected between the 12- and 0-hour groups (data not shown), and thus the 12-hour delay group was not pursued further.

The third hypothesis was that the proportion of dural immune cells is dependent on SPGN innervation, and is more pronounced in females than males. Changes in the proportion of dural immune cells in rats that underwent SCGx by removal of the superior cervical ganglion bilaterally (Figure 1b) were assessed. Lastly, given that stress drives activity in the SPGN, our fourth hypothesis was that the stress-induced increase in dural immune cells in females is dependent on the presence of SPGN innervation.

Immune cells from each animal were quantified with flow cytometry and analyzed as a percentage of total live cells so that changes in one subtype of immune cell could be determined independently of changes in another cell type. The percentage of total live cells was normalized to the respective male naïve group in order to facilitate comparisons between groups.

Data were analyzed with *t*-tests for sex effects (male versus female), two-way analyses of variance (ANOVAs) for sex and stress effects ( naïve, 0, 12 or 24 hours following CVS) and sex and SPGN effects (intact versus SCGx) in naïve animals, and three-way ANOVAs for sex, stress, and SPGN effects, with *p*<0.05 considered significant. The Tukey HSD test was used for post-hoc comparisons if significant interactions were detected. An analysis of covariance was used in order to assess the effect of estrus cycle with uterus weight as an approximate measure of cycle stage (24).
Results

This study was completed in three parts. First, we confirmed that the CVS model was stressful. Second, we used flow cytometry to identify the immune cell types that are present in the dura and validated these data with complementary morphology following single-cell sorting and IHC of intact dura. Third, we used flow cytometry to determine the impact of sex, stress, and SPGN innervation on the relative proportions of dural immune cell subtypes.

CORT and weight changes associated with the CVS model

CVS was associated with a small but significant (p<0.01) elevation of plasma CORT in both male and female rats that persisted for 24 hours after stress was terminated (Figure 1c). Furthermore, weight gain in both males and females was halted (p<0.01) during the CVS exposure, but returned to normal over the 24-hour period following CVS (Figure 1d).

Immune cell types present in the dura

Flow cytometry, with the gating strategy illustrated in Figure 2, was used to characterize immune cell types. Within CD45+ leukocytes, six types of immune cells were detected (Figure 3), as assessed using antibodies previously validated in the rat (25–28). In the naïve male dura, 63.2±0.9% of immune cells were CD11b+ macrophages/monocytes, granulocytes, and mast cells (“Macs”); 0.9±0.2% were CD11b/c+CD11b− dendritic cells (DCs); 20.0±1.0% were CD45R+ B-cells; 3.1±0.6% were CD161a+ natural killer (NK) cells; 4.5±0.4% were CD3+ T-cells; and 0.5±0.1% were CD161a+/CD3+ NK T (NKT) cells. An average of 7.8±0.3% of immune cells were not specifically identified in our panel (“unidentified”). This flow cytometry strategy was validated by FACS for the major subpopulations of immune cells, confirming their identity with morphological and histological analysis. Diff-Quik staining (Figure 3, insets) confirmed that Macs consisted of 89.1% macrophages/monocytes with a characteristic and pronounced granularity, 5.1% neutrophils, 3.6% mast cells (further identified with toluidine blue stain), and 2% contamination by lymphocytes. The B-cell population consisted of 93.0% small lymphocytes (≤10 μm) and 5.1% large lymphocytes (>10 μm), both of which were non-granular, with <2% contamination by macrophages/monocytes. The NK cell population consisted of 51.3% large and 48.7% small lymphocytes, of low granularity. The T-cell population consisted of 79.7% small and 20.3% large non-granular lymphocytes.

In order to exclude the possibility that immune cell estimates were confounded by incomplete perfusion of the dural vasculature, as well as to determine the distribution of major immune cell populations detected within the dura, Macs, NK cells, and T-cells were assessed in situ with ED-2-, CD161a-, and CD3-like immunoreactivity (-LI), respectively (Supplemental Figure 2). Together, these observations indicate that there is a large and diverse immune cell population in the dura.

Sex, stress, and sympathetic innervation influence dural immune cell proportions

Immune cells in the dura—While there was no detectable difference between naïve male (n=7) and female (n=6) rats in the proportion of total immune cells (CD45+) in the dura (Figure 4a, “Intact, naïve” groups), there was a significant (p<0.05) interaction between
sex and stress (Figure 4a, “Intact” groups). This was due to a stress-induced increase in dural immune cells present in males, but not females, which was significantly greater ($p<0.05$; $n=6$) immediately after stress (0 hours) and maintained ($p<0.05$; $n=8$) for 24 hours after stress.

There was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals regarding the proportion of immune cells in the dura (Figure 4a, “Intact, naïve” and “SCGx, naïve” groups). This was due to the significant ($p<0.05$) decrease in immune cells with SCGx in naïve females ($n=6$), although this may have been a surgery effect, as it was also seen in sham-operated rats (Figure 4b). There was also a significant ($p<0.05$) increase in immune cells with SCGx in naïve males ($n=6$), which was likely due to SPGN effects, as it was not seen in sham animals. Folding all additional groups into the analysis revealed a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation on dural immune cells (Figure 4a). It is important to note, however, that the loss of the stress effect in males with SCGx may have been a surgery effect based on sham animals (Figure 4b).

**Myeloid-derived dural immune cell subtypes**

**Macrophages/monocytes, granulocytes, and mast cells**—The proportion of Macs was significantly ($p<0.05$) higher in the dura from intact naïve females than intact naïve males. There was also a significant ($p<0.05$) interaction between sex and stress on the proportion of Macs in the dura (Figure 5a). This was due to a stress-induced increase in Macs present in males, but not females, which was significantly greater immediately after stress ($p<0.05$) and maintained with a 24-hour delay after stress ($p<0.01$) compared with naïve rats. In addition, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve rats on the proportion of Macs in the dura (Figure 5a). The increase in Macs in naïve SCGx males was significant ($p<0.05$), while there was only a trend ($p=0.056$) toward a decrease in Macs in naïve SCGx females. Notably, while the trend in the decrease in naïve SCGx females was observed in the sham surgery group, the significant increase in Macs in naïve SCGx males was not, and is therefore likely due to SPGN effects (Figure 5b). Lastly, there was a significant ($p<0.05$) interaction between sex, stress, and SPGN innervation regarding Macs in the dura (Figure 5a). However, the loss of a stress effect with SCGx was also seen in sham-operated rats (Figure 5b), and may be due to surgery effects.

**Dendritic cells**—There was a significant ($p<0.05$) interaction between sex and stress on the proportion of dural DCs (Figure 5c). This was due to a stress-induced increase in DCs present in males, but not females, which was significant ($p<0.001$) immediately after stress (Figure 5c). There was also a significantly ($p<0.05$) higher proportion of DCs in naïve males than females following SCGx (Figure 5c), which was not an effect of surgery (Figure 5d). Lastly, there was a significant ($p<0.05$) interaction between sex, stress, and SCGx regarding the proportion of dural DCs (Figure 5c). The stress-induced increase in DCs in SCGx females was significant ($p<0.001$) immediately after stress and was also not an effect of surgery, although the loss of the stress-induced increase at 0 hours in SCGx male rats may have been. Interestingly, in sham rats, the stress-induced increase in DCs was only significant with a 24-hour delay following stress (Figure 5d).
Lymphoid-derived dural immune cell subtypes

**T-cells**—There was a significant ($p<0.05$) interaction between sex and stress (Figure 6a) regarding the proportion of dural T-cells. This was due to a stress-induced increase in T-cells that was present in females, but not males, which was significantly greater 24 hours after stress than immediately after stress ($p<0.05$) or in the naïve rats ($p<0.01$). In addition, there was a significant main effect ($p<0.05$) of SPGN innervation (Figure 6a) due to the larger proportion of T-cells in the dura for SCGx than intact rats. Lastly, the sex and stress interaction and SPGN main effect persisted with all groups folded into the analysis. However, the overall increase in T-cells with SCGx was also seen in sham-operated rats (Figure 6b) and may be due to surgery effects.

**NKT cells**—The variability was large for the minor subgroup of T-cells – NKT cells – likely due to the overall low number of NKT cells in the dura. Nevertheless, there was a significant ($p<0.05$) main effect of sex on the proportion of NKT cells in the “Intact” groups (Figure 6c). This was due to a smaller proportion of NKT cells in females than in males. This overall pattern was maintained in SCGx rats, and as a result, SPGN innervation had no significant effect on NKT cells.

**NK cells**—The variability was considerably larger for NK cells than any other immune cell type studied. Nevertheless, we were still able to detect a significant ($p<0.05$) sex by stress interaction when all groups were folded into the analysis (Figure 6e). This was due to a significant ($p<0.001$) increase in NK cells immediately after stress in females. There were no SCGx effects.

**B-cells**—The relative proportion of B-cells was significantly ($p<0.05$) lower in the dura from intact naïve females than males (Figure 6g). This sex difference persisted as a main effect ($p<0.05$) after folding stress groups into the analysis (Figure 6g). In addition, when folding in all groups, there was a significant ($p<0.05$) main effect of SPGN innervation (Figure 6g). This was not likely due to surgery effects, as the decrease in the proportion of B-cells across all SCGx groups was not detected in the sham surgery groups (Figure 6h).

**“Unidentified” dural immune cell subtypes**—When all groups were folded into the analysis, the proportion of “unidentified” immune cells in females was significantly ($p<0.05$) greater than in males (Figure 7a).

**Discussion**

The purposes of this study were twofold: 1) to characterize the identity and proportions of immune cells present in the dura; and 2) to determine how the proportions of immune cells in the dura were influenced by sex, stress, and/or SPGN innervation. A combination of flow cytometry, IHC and FACS of rat dura indicated that ~17% of the total cells in the dura were immune cells, generally comparable to the colon (~20% in our preliminary studies; data not shown), a highly immune-competent tissue (29,30). Dural immune cells were composed of at least six subtypes: myeloid-derived Macs and DCs and lymphoid-derived T-, NKT, NK, and B-cells, identified by lineage-specific cell surface markers, morphology, and histology. These six immune cell types accounted for ~92% of the total dural immune cells. The small
subset of “unidentified” immune cells may have been comprised of CD11b− DCs, CD11b− mast cells, and/or immune cell progenitors that had not yet differentiated/matured into peripheral tissue leukocytes. Having confirmed that CVS produced a stress response, we found that the relative proportions of these immune cell types were influenced by sex, stress, and SPGN innervation. There were significantly more myeloid-derived Macs and less lymphoid-derived B- and NKT cells in the dura of females compared with males. Both Macs and DCs were increased by stress in males but not females, and this increase was maintained for at least 24 hours following stress for the Macs. In contrast, lymphoid-derived T-cells were significantly increased in females with a 24-hour delay following stress.

Previous data indicate that the dura is enriched with resident myeloid-derived mast cells (4,31–33) and macrophages (5,34–36). Our observations extend these previous results in three important ways. First, we quantified the relative proportion of resident immune cells in the dura. Second, we described the impacts of sex, stress, and SPGN innervation on the proportions of these cells. Third, our results clearly indicate that in addition to the classical resident immune cells, immune cells traditionally described as “recruited” are also present. Previously, lymphoid-derived immune cells have been described in the dura under pathological conditions such as meningitis (37–39) or tumors (40), but only recently also under “naïve” conditions (41). Because we minimized vascular contamination of immune cells by removing blood prior to tissue collection and demonstrated the presence of NK and T-cells in situ with IHC, our results strongly argue against the possibility that the presence of lymphoid-derived immune cells in the dura was an artifact.

Although it is still unknown whether the immune cell subtypes vary within regions of the dura, the localization of both lymphoid- and myeloid-derived immune cells near the vasculature and large nerve bundles (not shown) would enable these cells to play a role in the initiation and the resolution of migraine pain. We suggest that this is true for all the immune cell types detected, despite the relatively small numbers in which some types were present. Indeed, mast cell degranulation drives dural afferent activity (4), despite the fact that this subpopulation of immune cells appears to represent only ~2.4% of immune cells in the dura, as estimated by the results of our cytological analysis and toluidine blue staining (Figure 3).

While performed as a control, the results from the sham surgery groups were interesting in several respects. The response to sham surgery was dependent on immune cell type. If present, sham surgery was associated with a decrease in the proportion of myeloid-derived subtypes and an increase in lymphoid-derived subtypes. There are clear examples of a stressor sensitizing or priming the immune system response to a subsequent stress (42,43), and this may explain the enhanced responses in the lymphoid-derived immune cell subtypes with sham surgery. However, surgery as a stressor is generally immunosuppressive for less than 1 week (44–47). That any effect would last for 12 days in the sham groups was unexpected. We acknowledge the possibility of other compensatory effects due to SCGx, although we (Figure 1b) and others (48) have not detected any surgery-induced changes in calcitonin gene-related peptide IHC. While the relative impact of SPGN innervation on stress-induced changes in dural immune cells was confounded by the influence of surgery, per se, there were still cases of marked differences between SCGx and sham surgery groups,
implicating a role of SPGN in the regulation of dural immune cells. This was most clearly evident in the proportion of Macs, DCs, and T-cells, which were significantly higher in SCGx-naïve males but not females.

Our observations suggest several new areas of investigation concerning the mechanisms underlying the impacts of sex, stress, and SPGN innervation on immune cells in the dura. Given the evidence of gonadal hormone regulation of immune cells (7,8,49), it will be important to determine the extent to which the regulation of immune cells in the dura is dependent on gonadal hormones. In order to begin to address this issue, we analyzed uterus weights as an indirect measure of cycling in the rats (24). Although a subtype of migraine is correlated with the menstrual cycle in humans, we did not find correlations between any immune cell subtypes and the uterus weights of rats, suggesting that hormone fluctuations associated with the estrus cycle in female rats were not likely to be responsible for the sex differences observed in the dura. Second, with the additional evidence of adrenergic regulation of immune cells, it will be important to determine whether norepinephrine is the mediator that is primarily responsible for the impact of SPGN innervation on immune cells. However, given our evidence of several immune cell types being influenced by stress but not SPGN innervation or in which there was an interaction between stress and SPGN innervation, additional mediators are likely to contribute to the regulation of dural immune cells. For example, mediators released from the primary afferents themselves are also likely to play a role.

Third, the full implications of the dynamic regulation of dural immune cells will ultimately require functional analysis. In order to begin to address this issue, we recently reported (50) that there was a delayed stress-induced shift from anti- to pro-inflammatory mediator expression in female dural myeloid-derived cells. This is suggestive of a shift to an M1 phenotype in females. There was also a delayed stress-induced increase in pro- and anti-inflammatory mediator expression in female dural lymphoid-derived cells. In combination with the changes in immune cell proportion described in the present study, these observations are consistent with changes that may set the stage for a migraine attack. As we did not propose to create a model of migraine or to have given a migraine to the rats in this study, it will be important to determine whether and how these changes in immune cells are engaged by additional factor(s) that must be necessary to tip the balance either towards the initiation of an attack or towards the normalization of immune cells in the dura in the absence of an attack.

Nevertheless, the changes observed in this study have at least two important implications. First, the observation that there is a sex difference in the stress-induced increase in dural immune cells suggests that it may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women. Second, our observations provide the first evidence implicating T-cells in migraine pathogenesis. We suggest this because: 1) migraine is associated with sterile inflammation of the dura; 2) stress can trigger a migraine attack, but only after a delay following the termination of the stress; and 3) migraine is more prevalent in females. Our observation that stress can drive an increase in T-cells in the dura, but only after a delay and only in females, indicates that there may be an association between T-cells and migraine.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References


The composition of immune cells in the dura in the absence of pathology is far more complex than originally appreciated.

The relative distribution of immune cell types within the dura is influenced by sex, stress, and sympathetic post-ganglionic neuron innervation, as well as a significant interaction between these three factors.

The presence of a sex difference in the stress-induced increase in dural immune cells suggests that it may not only be possible, but necessary to use different strategies for the most effective treatment of migraine in men and women.

T-cells may not only account for the sex difference in the manifestation of migraine, but also the delay in the initiation of a migraine attack following a period of stress.
Figure 1.

Experimental design. (a) Intact and SCGx male and female rats were either considered “naïve” or stressed with the “CVS” paradigm. Tissue was collected from CVS rats immediately after the last day of stress (“CVS+0 h”) as a measure of the response to chronic stress or following a 24-hour delay after stress (“CVS+24 h”) as a measure of the response to stress-relaxation. (b) To test the impact of sympathetic post-ganglionic neuron innervation, prior to stress, superior cervical ganglia were surgically removed bilaterally (SCGx) in the groups as indicated. On the left, a representative whole-mount dura from an adult rat 7 days after a unilateral SCGx is shown, probed with anti-CGRP (green) or anti-TH (red) antibodies. Note the complete loss of TH-like immunoreactivity (a marker for sympathetic fibers) ipsilateral to the SCGx, but no change in CGRP-like immunoreactivity (a marker for peptidergic afferents). (c) CVS is a mild stress paradigm. There was a significant (p<0.01) main effect of stress on plasma levels of CORT in rats. CORT was significantly (p<0.01) higher in rats exposed to CVS (naïve versus CVS+0 h), and this remained significantly (p<0.01) elevated with a delay after stress (naïve versus CVS+24 h). There was no significant (p>0.05) main effect of sex or interaction with stress. Note that the CVS paradigm produced a significantly (p<0.001) smaller increase in CORT compared with the potent, acute restraint stress paradigm. (d) In addition, there was a significant (p<0.001) main effect of stress on daily weight gain in rats. Rats gained significantly (p<0.05) less weight per day during CVS (naïve versus CVS+0 h), which recovered (p<0.001) during the relaxation phase after stress (CVS+0 h versus CVS+24 h).

CGRP: calcitonin gene-related peptide; CORT: corticosterone; CVS: chronic variable stress; SCGx: surgical sympathectomy; TH: tyrosine hydroxylase.
Figure 2.
Gating strategy used for the isolation of immune cell subtypes from the dura. The data shown are from a naïve male rat. On the day of the experiment, dural cells were dissociated and stained with antibodies specific to markers of immune cells and/or distinct immune cell subtypes. The gating strategy illustrated was used for all flow and fluorescence-activated cell sorting experiments. CD45+ immune cells were first selected, and then sorted into CD11b+ macrophages/granulocytes/mast cells (“Macs”) and CD11c+DCs. CD11b−/c− immune cells were further sorted into CD45R+ B-cells. CD11b−/c− and CD45R− immune cells were lastly sorted into either CD161a+ NK cells, CD3+ T-cells, or CD161a+/CD3+ NKT cells. A small subset of immune cells was left unstained by any of the immune subtype antibodies that were utilized in this strategy (“unidentified”). Viability was uniformly 80–85%, as determined by propidium iodide and trypan blue staining. This resulted in an average of $1.56 \times 10^6 \pm 0.18 \times 10^6$ live cells recovered per dura. Spleen cells were used for compensation controls (51,52). Images are from FlowJo software, contour plots were used with 5% levels and outliers displayed.

DC: dendritic cell; NK: natural killer; FSC-A: forward scatter.
Figure 3.
Proportion of immune cell subtypes in the naïve male dura as determined with flow cytometry using the gating strategy shown in Figure 2. Of the live cells recovered from the dura, an average of 16.9±0.90% were CD45+, and thus determined to be immune cells. The pie chart and legend show the relative proportions of the six immune cell subtypes identified in the dura. The insets show fluorescence-activated cell sorted, cyto-centrifuged preparations of immune cell subtypes, subsequently stained with Diff-Quik. Macs consisted of 89.1% macrophages/monocytes (M), 5.1% neutrophils (N), 3.6% mast cells (MC; identified with toluidine blue stain), and 2.2% lymphocytes. The NK cell population consisted of 51.3% large lymphocytes (>10 μm) and 48.7% small lymphocytes (≤10 μm). The T-cell population consisted of 79.7% small lymphocytes and 20.3% large lymphocytes. Lastly, the B-cell population consisted of 93.0% small lymphocytes, 5.1% lymphocytes, and 1.9% macrophages/monocytes. Scale bars: 10 μm.
DC: dendritic cell; Macs: macrophages/granulocytes; NK: natural killer.
Figure 4.
Sex-, stress-, and SPGN-dependent changes in the percentage of CD45+ immune cells in the dura (relative to the total number of live cells recovered). Data are presented as percentages of naïve males in order to facilitate comparisons between groups. (a) There was a significant interaction between sex and stress regarding the proportion of live immune cells in the dura ($p<0.05$) (“Intact” groups). In addition, there was a significant ($p<0.05$) interaction between sex and SPGN innervation in naïve animals regarding the proportions of immune cells in the dura (“Intact, naïve” and “SCGx, naïve” groups). Lastly, there was an interaction between sex, stress, and SPGN innervation ($p<0.05$) regarding the proportion of immune cells in the dura. (b) Similar to male SCGx groups, male sham-operated rats also showed the loss of a stress-induced increase in immune cells.

H1=a priori hypothesis 1: sex difference in “Intact, naïve” groups, analyzed with a t-test; H2=a priori hypothesis 2: sex×stress comparison in “Intact” groups, analyzed with a two-way analysis of variance (ANOVA); H3=a priori hypothesis 3: sex×SCGx in “Naïve” groups (i.e. “Intact” versus “SCGx” naïve), analyzed with a two-way ANOVA; H4=a priori hypothesis 4: sex×stress SCGx interaction between all groups, analyzed with a three-way ANOVA.

*p<0.05.

CVS: chronic variable stress; SCGx: surgical sympathectomy; SPGN: sympathetic post-ganglionic neuron.
Figure 5. Sex-, stress-, and SPGN-dependent changes in the percentage of myeloid-derived immune cells dissociated from the dura (relative to the total number of live cells from each dura). Data are presented as percentages of naïve males for comparison. (a) The proportion of Macs was significantly (p<0.05) higher in the dura from intact naïve females than males. There was a significant (p<0.05) interaction between sex and stress regarding the proportion of Macs in the dura (“Intact” groups). There was also a significant (p<0.05) interaction between sex and SPGN innervation in naïve rats regarding the proportion of Macs in the dura. (b) The proportion of DCs was significantly (p<0.05) higher in the dura from intact naïve males than females. There was a significant (p<0.05) interaction between sex and stress regarding the proportion of DCs in the dura (“Intact” groups). There was also a significant (p<0.05) interaction between sex and SPGN innervation in naïve rats regarding the proportion of DCs in the dura.
dura ("Intact, naïve" and "SCGx, naïve" groups). Lastly, there was an interaction between sex, stress, and SPGN innervation ($p<0.05$). (b) Similar to male SCGx groups, male sham-operated rats also showed the loss of a stress-induced increase in Macs. (c) There was a significant ($p<0.05$) interaction between sex and stress on the proportion of dural DCs ("Intact" groups). In addition, there was a significant ($p<0.05$) interaction between sex, stress, and SCGx. (d) Interestingly, in sham rats, the stress-induced increase in DCs was only seen with a delay following stress. The groups and a priori hypotheses (H1–4) tested were the same as those in Figure 4.

*p<0.05; **p<0.01.

CVS: chronic variable stress; DC: dendritic cell; Macs: macrophages/granulocytes; SCGx: surgical sympathectomy; SPGN: sympathetic post-ganglionic neuron.
Figure 6.
Sex-, stress-, and SPGN-dependent changes in the percentage of lymphoid-derived immune cells dissociated from the dura (relative to the total number of live cells from each dura). Data are presented as percentages of naïve males for comparison. (a) There was a significant (p<0.05) interaction between sex and stress regarding the relative proportions of T-cells (“Intact” groups). In addition, there was a significant main effect (p<0.05) of SPGN innervation in naïve rats. Lastly, the sex and stress interaction and SPGN main effect persisted with all groups folded into the analysis. (b) Comparable stress-induced changes in T-cells were observed in sham surgery groups and in the SCGx groups. (c) There was a main effect of sex (p<0.05) on the proportion of NKT cells. (d) Sham-operated groups showed a stress effect in males, which may have been masked by SCGx. (e) There was a significant
(p<0.05) sex and stress interaction when all groups were folded into the analysis of NK cells. (f) Sham surgery did not appear to affect NK cells. (g) The proportion of B-cells was significantly (p<0.001) lower in the dura from intact naïve females than males, and this sex difference persisted as a main effect (p<0.05) after folding stress groups into the analysis (“Intact” groups). In addition, when all groups were folded in, there was a significant (p<0.05) main effect of SPGN innervation. (h) Sham surgery did not appear to affect B-cells. The groups and a priori hypotheses (H1–4) tested were the same as those in Figure 4.

*p<0.05; **p<0.01.

CVS: chronic variable stress; DC: dendritic cell; NK: natural killer; SCGx: surgical sympathectomy; SPGN: sympathetic post-ganglionic neuron.
Figure 7.
Sex-, stress-, and SPGN-dependent changes in the percentage of unidentified immune cells dissociated from the dura (relative to the total number of live cells from each dura). Data are presented as percentages of naïve males for comparison. (a) With all groups folded into the analysis, there was a main effect of sex (p<0.05) on the proportion of “unidentified” immune cells. (b) Sham surgery did not appear to affect “unidentified” cells. The groups and a priori hypothesis (H4) tested were the same as those in Figure 4. CVS: chronic variable stress; SCGx: surgical sympathectomy; SPGN: sympathetic post-ganglionic neuron.