Research Paper

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Brain networks and endogenous pain inhibition are modulated by age and sex in healthy rats

Joyce T. Da Silva[a,b,*], Christina Tricou[a], Youping Zhang[a], David A. Seminowicz[a], Jin Y. Ro[a]

Abstract

Endogenous pain inhibition is less efficient in chronic pain patients. Diffuse noxious inhibitory control (DNIC), a form of endogenous pain inhibition, is compromised in women and older people, making them more vulnerable to chronic pain. However, the underlying mechanisms remain unclear. Here, we used a capsaicin-induced DNIC test and resting-state functional MRI to investigate the impact of aging and sex on endogenous pain inhibition and associated brain circuitries in healthy rats. We found that DNIC was less efficient in young females compared with young males. Diffuse noxious inhibitory control response was lost in old rats of both sexes, but the brain networks engaged during DNIC differed in a sex-dependent manner. Young males had the most efficient analgesia with the strongest connectivity between anterior cingulate cortex (ACC) and periaqueductal gray (PAG). The reduced efficiency of DNIC in young females seemed to be driven by widespread brain connectivity. Old males showed increased connectivity between PAG, raphe nuclei, pontine reticular nucleus, and hippocampus, which may not be dependent on connections to ACC, whereas old females showed increased connectivity between ACC, PAG, and more limbic regions. These findings suggest that distinct brain circuitries including the limbic system may contribute to higher susceptibility to pain modulatory deficits in the elderly population, and sex may be a risk factor for developing age-related chronic pain.

Keywords: Aging, Sex, Brain, Pain, Descending pathways, Pain inhibition

1. Introduction

Chronic pain prevalence increases sharply with age and impacts physical and cognitive function, ultimately decreasing quality of life.[17] Risk factors for chronic pain in older people include female sex, psychosocial comorbidities, increased pain facilitation, and diminished descending pain inhibitory capacity.[29] Without any clinical history of age-related neurological disorders, aging itself contributes to changes in brain structure and function, which affect pain processing.[5]

Accordingly, animal studies show that aging is associated with increased pain sensitivity, reduced analgesic effect of morphine, and higher risk for developing chronic pain.[3,26] Potential mechanisms for these phenomena are that advancing age results in enhanced nociceptor excitability[24] and a decline in endogenous inhibitory control of mu- and delta-opioid receptors in the spinal cord.[4] To the best of our knowledge, one basic science study investigated supraspinal pain mechanisms related to natural aging and found that molecular changes in the amygdala lead to higher pain-related behaviors in old male mice after inflammatory pain.[39] These available data suggest an apparent dysregulation of pain processing and pain modulation with aging at all levels of neuraxis.

We have previously demonstrated that endogenous pain inhibition and brain networks are modulated in a sex-dependent manner in rats.[7] Our paradigm assessed the diffuse noxious inhibitory control (DNIC) or the pain-inhibits-pain phenomenon, which has been extensively reported in animals and humans under healthy and chronic pain conditions.[34,35] However, our study innovatively led to the characterization of a model for associating brain regions with behavioral responses under a DNIC paradigm that is regulated by sex. Compared to females, males show increased connectivity between anterior cingulate cortex (ACC) and periaqueductal gray (PAG), and enhanced analgesic response induced by DNIC, which indicate a stronger descending pain inhibition in the presence of testosterone. By contrast, compared to males, females had increased ACC connectivity with hippocampal and thalamic regions, which was associated with reduced efficiency of pain modulation. These findings suggest that ACC plays a key role in descending modulatory pathways that affect pain response.

Although we documented sex differences in pain inhibition and brain networks in healthy rats, there is a lack of understanding of age-related effects on endogenous pain modulation and brain function. In this study, we combined DNIC behavioral testing and functional magnetic resonance imaging (fMRI) to assess age- and sex-dependent alterations in endogenous pain inhibition. Diffuse noxious inhibitory control responses and whole brain connectivity to ACC and PAG were assessed in young (3-6 months) and aged (20-24 months) male and female Fischer 344 rats. Our findings show that young males have the most efficient endogenous pain

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inhibition due to the strongest connectivity between ACC and PAG, whereas connections to the limbic system and distinct brain regions may contribute to higher susceptibility to pain modulatory deficits in young females and the elderly population. We further propose a model to point to the possibility of targeting specific brain regions in age- and sex-dependent painful conditions.

2. Methods

2.1. Animals

Fischer-344 rats consisting of young male (YM; 3-6 months old—250-290 g), young female (YF; 3-6 months old—160-175 g), old male (OM; 20-24 months old—405-455 g), and old female (OF; 20-24 months old—230-250 g) rats were obtained from the National Institute on Aging. Although the exact relationship between age of rats and age of humans is unknown, we approximate that young rats in our study would be comparable to 18-year-old humans, and old rats comparable to 60-year-old humans.[41] Animals were housed in a temperature-controlled room under a 12:12 light–dark cycle with access to food and water ad libitum. Rats in the same experimental groups were housed together in groups of 2 or 3. Male and female rats were housed together with similarly aged cage mates of the same sex in the same colony room, and each experimental group was tested at a different time. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under a University of Maryland-approved Institutional Animal Care and Use Committee protocol. For DNIC behavioral assay, capsaicin was administered to YM, YF, OM, and OF rats. A control injection of phosphate-buffered saline was made to age- and sex-matched rats. In an fMRI study, we investigated DNIC effects after capsaicin injection in separate groups of YM, YF, OM, and OF rats. All rats were randomly assigned to experimental and control groups (n = 5).

2.2. Drug preparation and administration

Capsaicin (Millipore Sigma, St. Louis, MO) was dissolved in ethanol (20%), Tween 20 (7%), and phosphate-buffered saline (93%). For behavioral studies, capsaicin (0.3% in 100 μL) was administered once intradermally in the left forepaw using a 27-gauge needle. The same volume of vehicle was injected in the same manner in control animals. For fMRI studies, the same concentration of capsaicin was administered in the left hind paw as described previously.

2.3. Behavioral assay and statistical analysis

The model of DNIC in this study was adapted from our previous study in rats.[7] Hind paw withdrawal latencies to noxious thermal stimulation, a test stimulus, were measured before and 15, 30, 45, 60, 90, and 120 minutes after the administration of capsaicin, a conditioning stimulus, into the left forepaw. Hind paw withdrawal latencies to a thermal nociceptive stimulus were assessed according to the methods described in a previous study (Hargreaves et al., 1988). Rats were allowed to habituate to the experimental room for 30 minutes per day for 3 consecutive days. Rats were placed on an elevated glass surface and allowed to acclimate for 10 to 20 minutes. A radiant heat source was directed to the plantar surface of the hind paw from underneath the glass floor. A motion detector halted both lamp and timer when the paw was withdrawn. The voltage of the bulb was adjusted to result in an average paw withdrawal latency of 10 to 12 seconds in naive animals. A 20-second cutoff was used to prevent tissue damage. Three trials (with an intertrial interval of at least 5 minutes) were determined for each hind paw, and the average of the trials was used as the mean thermal paw withdrawal latency. The increase in hind paw withdrawal latency after capsaicin treatment in the forepaw was used as the measure of DNIC. To assess the overall magnitude of drug-induced changes in DNIC over time, area under the curve (AUC) was calculated for the normalized data for each rat using the trapezoid rule.

Results were analyzed using the statistical analysis software package SigmaPlot. Two-way repeated-measures analysis of variance (ANOVA) with Holm–Sidak method for correction of multiple comparisons were performed to determine significant treatment and time effects for each age and sex group. Two-way ANOVA was used to compare AUC across different age and sex groups treated with either capsaicin or vehicle. Differences were considered statistically significant at $P < 0.05$ and the data were presented as mean ± SEM. The investigators conducting the behavioral study were blinded to the experimental groups and drug administration, ie, animals that received either capsaicin or vehicle.

2.4. Resting-state functional magnetic resonance imaging data acquisition

Data were acquired using a Bruker BioSpec 70/30USR Avance III 7-Tesla scanner (Bruker Biospin MRI GmbH, Ettlingen, Germany) and a 40-mm circular polarized volume coil. During scanning, rats were anesthetized at a constant mixture of 1.5% isoflurane in oxygen-enriched air, and respiration and heart rate were monitored with a small animal monitoring and gating system and software (SA Instruments, Inc, Stony Brook, NY). T2-weighted images were obtained using a 2D RARE (400 × 400 matrix, 22 coronal 1-mm slice thickness, in plane resolution 100 μm, TR 2000 ms, TE 28 ms). rsfMRI scans were acquired using an echo planar imaging sequence (TR 1500 ms, TE 24 ms, 128 × 128 matrix, in plane resolution 0.40 × 0.40 × 1 mm, 22 coronal slices, 620 volumes per scan). The anatomical and the first resting-state functional magnetic resonance imaging (rsfMRI) scans were performed for each rat as baseline (before capsaicin injection, 15 minutes). Rats subsequently received an injection of capsaicin (0.3% in 100 μL) into the left hind paw, and 3 rsfMRI scans were acquired for 15 minutes each (**Fig. 1**). The investigators analyzing the MRI data were blinded to the experimental groups. Investigators running the MRI sessions were not blinded to the experimental groups due to visible sex and weight differences between animals.

2.5. rsfMRI preprocessing, statistical analysis, and data availability

All preprocessing and analyses were performed in SPM12 (http://www.fil.ion.ucl.ac.uk/spm/). We used seed-based analysis to assess how functional connectivity (FC) between right anterior cingulate cortex (R ACC) or PAG and the whole brain varies after capsaicin injection between the different groups. The contralateral side to capsaicin injection was used. We selected ACC and PAG as regions of interest (ROIs) based on prior literature showing changes in connectivity and neuronal activation of these areas during descending pain inhibition, which was also modulated by sex.[7,30] Anatomical locations were chosen according to Paxinos and Watson atlas (2004). We first created a study-specific template by coregistering and averaging the T2-weighted images across animals and interpolating to voxel size of isotropic 0.5 mm. Preprocessing steps included slice timing correction (number of slices: 22, reference slice: 11), realignment and motion correction (separation: 0.57, smoothing—full width at

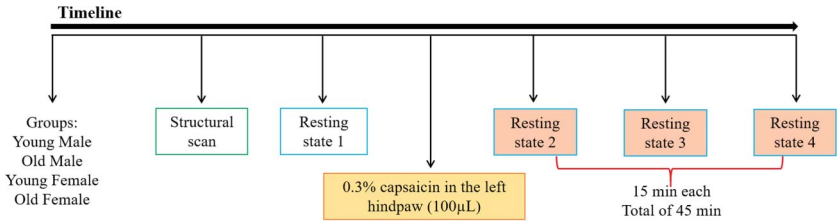

Figure 1. Schematic illustrating the fMRI experimental design. All groups underwent structural scan (green box), 1 resting-state scan before capsaicin injection (blue box—baseline 15 minutes), capsaicin injection and 3 resting state scans of 15 minutes each (orange filled boxes—post-capsaicin 15, 30 and 45 minutes). The total time of resting-state scans after capsaicin injection was 45 minutes. fMRI, functional magnetic resonance imaging.

half maximum: 0.76, interpolation for reslicing: fourth Degree B-Spline), within-subject registration (separation: [1 0.5], histogram smoothing: [1.7 1.7]), normalization to the study-specific template (source imaging smoothing: 1.52), normalization of functional images (0.5 isotropic voxel, interpolation: trilinear), bandpass filtering (0.009-0.2 Hz), and smoothing at 1-mm full width at half maximum. We then extracted time series data from the ROIs and regressed these time courses with the signal at each voxel across the whole brain to reveal FC patterns for each animal. Six motion parameters were included as regressors of no interest. To assess the effects of capsaicin on resting-state connectivity, the first-level β-contrast images representing ACC and PAG FC for each of the 4 time points (baseline resting state and 3 resting-state scans after capsaicin) were entered as dependent variables in the general linear model for each rat within each group. Second-level analyses were performed according to the following 3 designs: (1) flexible factorial design to show age differences in ACC FC to the whole brain with 2 Group (young and old) × 4 Time (baseline and 3 resting state scans of 15 minutes each), with Group and Time specified as fixed factors. Comparisons were done between baseline and resting state 4 (from 30 to 45 minutes). (2) Flexible factorial design to show sex differences in ACC FC to the whole brain with 2 Group (male and female) × 4 Time (baseline and 3 resting state scans of 15 minutes each), with Group and Time specified as fixed factors. Comparisons were done between baseline and resting state 4. (3) Finally, 2 flexible factorial designs to examine the full age-by-sex interaction (YM, YF, OM, and OF) in ACC and PAG FC to the whole brain during DNIC comparing baseline vs resting state 4. Because of the small sample size and exploratory nature of the study, second-level maps used cluster-forming (voxel level) thresholds at $P < 0.05$, 0.01, 0.005, and 0.001. Significant clusters for each threshold were reported. Analyses described in 1 and 2 were also done in the entire 45 minutes of fMRI data after capsaicin injection, and in resting state 2 (0-15 minutes after capsaicin) and resting state 3 (15-30 minutes after capsaicin) separately. Results are reported in the supplemental material (available at http://links.lww.com/PAIN/A946). For visualization, we extracted and plotted the average beta values ± SEM from all significant clusters at $P < 0.05$ for each animal and time point. We note that the cluster-forming threshold of $P < 0.05$ is overly liberal but we present these results as exploratory. All data including code, ROIs, and the template brain are available upon reasonable request.

3. Results

3.1. Diffuse noxious inhibitory control impaired in young females and lost in old rats

Our previous study showed a sex-dependent modulation of the analgesia produced by capsaicin-induced DNIC, which we assessed with mechanical sensitivity testing in rats.[7] In the current study, DNIC was induced by capsaicin injection into the forepaw, as the conditioning stimulus, but thermal sensitivity was assessed on the hind paw, as a test stimulus, to determine age- and sex-related differences. Capsaicin, but not vehicle, resulted in significant increases in hind paw withdrawal latencies in young male rats (**Fig. 2A**; treatment effect $F = 179.7$, $P < 0.001$; time effect $F = 47.7$, $P < 0.001$). The increase in latency was observed as early as 15 minutes after capsaicin treatment. The significant increase was maintained at the cutoff limit of 20 seconds for 60 minutes before it gradually declined to the baseline level by 120 minutes. The vehicle administration in the forepaw in another group of young male rats did not alter the paw withdrawal latencies at any of the time points we observed. The capsaicin administration in young female rats also induced an immediate increase in hind paw withdrawal latencies (**Fig. 2B**. treatment effect $F = 21.06$, $P < 0.01$; time effect $F = 21.38$, $P < 0.001$). Although the increase in latency was significant up to first 45 minutes, unlike in young male rats, the capsaicin-induced latency began to decline within 30 minutes and reached the near baseline in 90 minutes. The vehicle administration in the forepaw in another group of young female rats did not alter the paw withdrawal latencies.

In old male rats, there was no significant difference in withdrawal latencies between capsaicin- and vehicle-treated rats (**Fig. 2C**; $F = 1.96$, $P > 0.05$). There was a significant main effect for time ($F = 7.65$, $P < 0.001$), which was primarily contributed by the increase in latency at the 15-minute time point. Similarly, no significant difference in withdrawal latencies between capsaicin- and vehicle-treated old female rats was observed (**Fig. 2D**; $F = 1.95$, $P > 0.05$). Both vehicle and capsaicin tended to increase the withdrawal latency at 15 minutes after injection, a time point at which significant differences was detected ($F = 7.6$, $P < 0.01$).

To compare the extent of DNIC across the two-hour time span, we compared AUC obtained from each age and sex groups. The two-way ANOVA revealed significant sex ($F = 108.7$, $P < 0.001$) and age (**Fig. 2E**; $F = 12.5$, $P < 0.01$) effects. Post hoc multiple group comparisons revealed that AUC obtained from young males treated with capsaicin was significantly greater than AUC from young females, old males, and old females under the same condition. Area under the curve from young females was significantly greater than AUC from old males and old females. There was no difference in AUC between old males and old females. There was no significant age or sex effect between any groups treated with the vehicle (**Fig. 2F**; $F = 0.42$, $P > 0.05$, $F = 0.03$, $P > 0.05$, respectively). These findings are consistent with our previous study showing that young males have a more efficient DNIC response compared to young females. The data also supported our hypothesis that DNIC is lost in old animals. Based on these observations, we hypothesized that brain connectivity related to DNIC response would be modulated in an age- and sex-dependent manner.

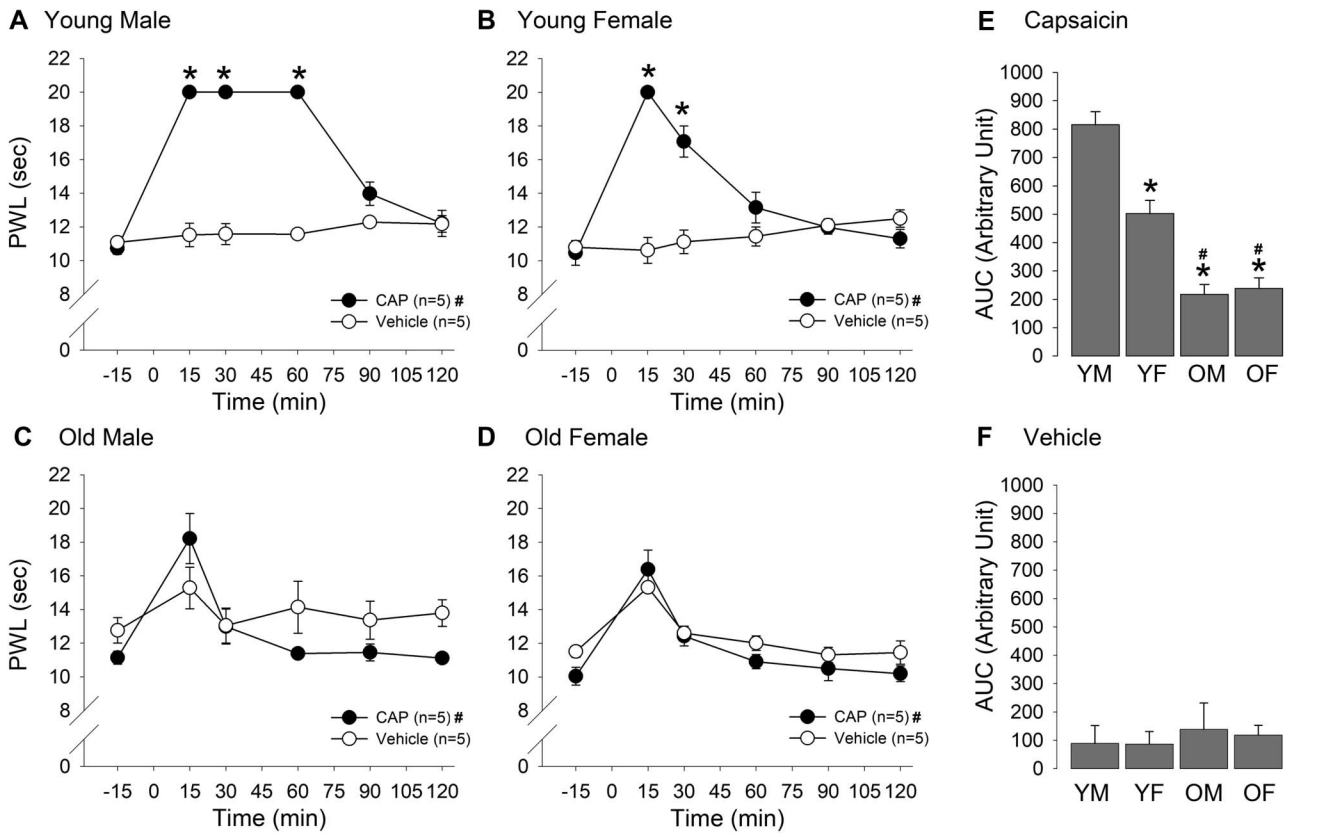

Figure 2. Age- and sex-related differences in DNIC. Changes in hind paw thermal withdrawal latencies to capsaicin and vehicle treatment in forepaw of young male (A), young female (B), old male (C), and old female (D) rats were plotted against time. *Significant main effect between treatments, and #Significant time effect. Bar graphs shown in (E and F) compare AUC as the measure of overall magnitude of DNIC between YM, YF, OM, and OF rats treated with capsaicin and vehicle, respectively. *Significant difference compared with YM rats, and #Significant difference compared with YF rats. AUC, area under the curve; DNIC, diffuse noxious inhibitory control.

3.2. Age- and sex-dependent changes in functional connectivity of anterior cingulate cortex after diffuse noxious inhibitory control induction

The age and sex effects on DNIC response were more robust 30 minutes after capsaicin injection, which suggest the use of capsaicin as a reliable conditioning stimulus at later time points. Therefore, we decided to explore the age and sex effects on ACC FC between 30 and 45 minutes (resting state 4) after DNIC induction. For age-dependent effects, young rats had increased right (R) ACC FC with anterior pretectal nucleus (APT), retrosplenial cortex (RS), deep mesencephalic nucleus (DpMe), and tegmental nucleus (Tg) compared with old rats (**Table 1** and **Fig. 3A**). Old animals had increased R ACC FC with hippocampus, raphe nuclei (Rn), pontine nuclei (Pn), and PAG relative to the young group (**Table 1** and **Fig. 3B**). For sex-dependent effects, males had increased R ACC FC with hippocampus, PAG, and DpMe compared to females (**Table 1** and **Fig. 3C**). Females had strong R ACC FC with RS, Pn, locus coeruleus (LC), paragigantocellular reticular nucleus (PGi), and gigantocellular reticular nucleus (Gi) (**Table 1** and **Fig. 3D**), relative to males. Because we also performed the full factorial analysis (supplemental material, available at http://links.lww.com/PAIN/A946), stronger age- and sex-dependent effects observed during the entire fMRI acquisition seem to be driven by resting state 4 (30-45 minutes after capsaicin). This phenomenon may occur due to the decrease of the initial nociceptive effects of capsaicin over time, which may suggest the use of capsaicin as a reliable conditioning stimulus at later time points. Thus, results from the entire postcapsaicin fMRI acquisition, resting state 2 (0-15 minutes after capsaicin), and resting state 3 (15-30 minutes post-capsaicin) are reported in the supplemental material to avoid massive amount of data (available at http://links.lww.com/PAIN/A946). Consequently, to investigate R ACC FC related to DNIC in a homogeneous sample of rats at a later time point, we decided to further stratify the groups by age and sex.

3.3. Functional connectivity of anterior cingulate cortex in groups stratified by age and sex after diffuse noxious inhibitory control induction

To advance our previous observations, we further stratified the animal samples by age and sex and investigated specific patterns of ACC connectivity during DNIC. For sex-matched groups showing effects of age, young females had increased R ACC FC with RS, DpMe, Tg, and additional connections to parietal association cortex (Pt) and cerebellum compared with old females (**Table 1** and **Fig. 4A**). Old females had increased R ACC FC with hippocampus, Rn, Pn, PAG, and additional connections to subiculum (Sub) relative to young females (**Table 1** and **Fig. 4B**). Right anterior cingulate cortex FC did not differ between young males and old males. For age-matched groups showing effects of sex, young males had increased R ACC FC with hippocampus, PAG, and DpMe compared to young females (**Table 1** and **Fig. 4C**). Young females had increased R ACC FC with RS, Pn, LC, PGi, cerebellum (**Table 1** and **Fig. 4D**), and additional connections to Pt relative to young males. Right

Table 1

Summary of the analysis approach and outcomes.

Analysis	Objective	Outcomes
1. Flexible factorial design with 2 groups (young and old) × 4 time points (baseline and 3 resting state scans). Comparisons between baseline and resting state 4.	To show age differences in ACC FC with the whole brain during DNIC.	Young group had increased ACC FC with the following network compared to old group (Fig. 3A): APT, RS, DpMe, and Tg. Old group had increased ACC FC with the following network compared to young group (Fig. 3B): PAG, hippocampus, Rn, and Pn.
2. Flexible factorial design with 2 groups (male and female) × 4 time points (baseline and 3 resting state scans). Comparisons between baseline and resting state 4.	To show sex differences in ACC FC with the whole brain during DNIC.	Males had increased ACC FC with the following network compared to females (Fig. 3C): PAG, hippocampus, and DpMe. Females had increased ACC FC with the following network compared to males (Fig. 3D): RS, Pn, LC, PGi, and Gi.
3. Flexible factorial design with 4 groups (YM, YF, OM, and OF) and comparisons between baseline and resting state 4 for ACC FC.	To examine the full age-by-sex interaction in ACC FC with the whole brain during DNIC.	Effects of age in sex-matched groups YF had increased ACC FC with the following network compared to OF (Fig. 4A): RS, DpMe, Tg, Pt, and cerebellum. OF had increased ACC FC with the following network compared to YF (Fig. 4B): PAG, hippocampus, Rn, Pn, and Sub. No differences between YM and OM. Effects of sex in age-matched groups YM had increased ACC FC with the following network compared to YF (Fig. 4C): PAG, hippocampus, and DpMe. YF had increased ACC FC with the following network compared to YM (Fig. 4D): RS, Pn, LC, PGi, cerebellum, and Pt. No differences between OM and OF.
4. Flexible factorial design with 4 groups (YM, YF, OM, and OF) and comparisons between baseline and resting state 4 for PAG FC.	To examine the full age-by-sex interaction in PAG FC with the whole brain in old males during DNIC.	Effects of age in sex-matched groups OM had increased PAG FC with the following network compared to YM (Fig. 5A): Hippocampus, APT, and RS. Effects of sex in age-matched groups OM had increased PAG FC with the following network compared to OF (Fig. 5B): Hippocampus, RS, Rn, Rt, Pn, and LC.

ACC, anterior cingulate cortex; APT, anterior pretectal nucleus; DNIC, diffuse noxious inhibitory control; DpMe, deep mesencephalic nucleus; FC, functional connectivity; Gi, gigantocellular reticular nucleus; LC, locus coeruleus; OF, old female; OM, old male; PAG, periaqueductal gray; PGi, paragigantocellular nucleus; Pn, pontine nuclei; Pt, parietal association cortex; Rn, raphe nucleus; RS, retrosplenial cortex; Rt, pontine reticular nucleus; Sub, subiculum; Tg, tegmental area; YF, young female; YM, young male.

anterior cingulate cortex FC did not differ between old males and old females. These results support the importance of age- and sex-matched groups to investigate connectivity of endogenous pain modulatory systems.

3.4. Functional connectivity of periaqueductal gray in groups stratified by age and sex after diffuse noxious inhibitory control induction

There was an increased ACC FC with PAG in the old group relative to young group with male and female rats combined. However, this effect was not seen in sex-matched groups when old males were compared with young males. Therefore, we decided to investigate the PAG connectivity to the whole brain in old males. We hypothesized that old males would distinctively recruit PAG connections during DNIC response, which may not be ACC-dependent. For sex-matched groups showing effects of age, old males had increased PAG FC with hippocampus, APT, and RS compared to young males (**Table 1** and **Fig. 5A**). For age-matched groups showing effects of sex, old males had increased PAG FC with hippocampus, RS, Rn, pontine reticular nucleus (Rt), Pn, and LC (**Table 1** and **Fig. 5B**), relative to old females. Thus, considering that PAG connectivity in old males differs from both young male and old female groups, this seems to be a unique mechanism in old males. This effect may be driven, at least in part, by the deficits observed in DNIC responses of old males compared with young males. It is worth noting that although the group differences in FC were larger at baseline, the opposite direction of the FC effects after DNIC may indicate a differential engagement of brain networks in each group, eg, old males had increased FC, whereas young male and old female had decreased FC during DNIC.

4. Discussion

Human studies have shown that the age-related decline in endogenous analgesia is due to reduced efficacy of descending inhibitory systems.[25,29,46] Consistent with these findings, we show that endogenous pain inhibition is lost in old rats and altered connectivity of brain regions associated with DNIC efficiency (**Fig. 6**). Although the analgesic effects of DNIC are lost in both old males and females, the brain networks engaged after the conditioning stimulus differ in a sex-dependent manner

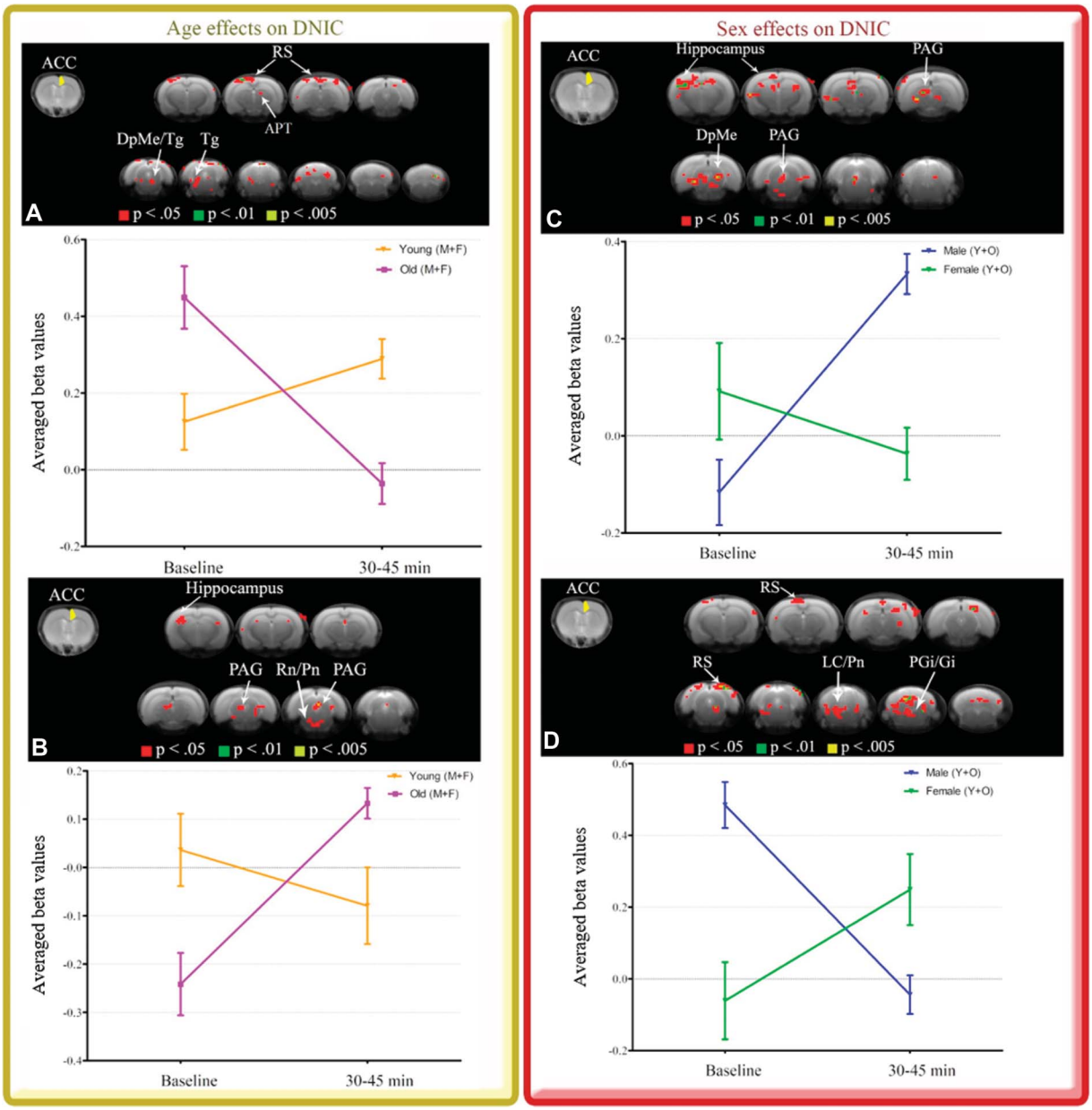

Figure 3. Right anterior cingulate cortex seed (yellow) and maps indicating connectivity to the whole brain during 30 to 45 minutes after DNIC induction. Effects of age (A and B) and sex (C and D) are illustrated. Brain images show cluster-forming thresholds at $P < 0.05$, 0.01, and 0.005. Plots show extracted beta values from all significant clusters thresholded at $P < 0.05$ for each animal and time point (average ± SEM). APT, anterior pretectal nucleus; DNIC, diffuse noxious inhibitory control; DpMe, deep mesencephalic nucleus; Gi, gigantocellular reticular nucleus; LC, locus coeruleus; PAG, periaqueductal gray; PGi, paragigantocellular nucleus; Pn, pontine nuclei; R ACC, anterior cingulate cortex; Rn, raphe nuclei; RS, retrosplenial cortex; Tg, tegmental nucleus.

suggesting that sex may be a risk factor for developing age-related chronic pain associated with compromised DNIC.

We showed that in old rats, DNIC induction through capsaicin as the conditioning stimulus did not affect paw withdrawal latencies, suggesting inefficiency of descending inhibitory systems. Capsaicin is known to produce ongoing and dose-dependent pain.[42] However, it is worth noting that the dose of capsaicin we used induces similar nocifensive responses and c-Fos activation in the spinal cord of female and male rats regardless of age.[36] Furthermore, capsaicin-induced pain decays exponentially after 15 minutes.[15] We assessed the same dose of capsaicin as a reliable conditioning stimulus for 120 minutes of behavioral testing and 45 minutes of fMRI acquisition. Capsaicin-induced analgesia from young rats was seen as early as 15 minutes and remained for at least 45 minutes, which likely represents DNIC. Therefore, the effects of capsaicin on central processing through descending pain modulation seems to last longer than its effects on primary afferent nociceptors, which ceases in a relatively short period.[42]

The ACC lies in a unique position in the brain, with connections to the limbic system and top-down influences on the PAG to gate pain modulation.[43] In this study, the fMRI data from 30 to 45 minutes after capsaicin injection captured several age- and sex-related changes in ACC connectivity, which had stronger effects and exclusion of potential false-positive voxels compared with findings from earlier time points. A possible explanation for these findings is that the reduction of the initial nociceptive effects of capsaicin over time could, to some extent, justify the use of capsaicin as a reliable conditioning stimulus at later time points.[42] Regardless of sex, we found that age is a crucial factor to change

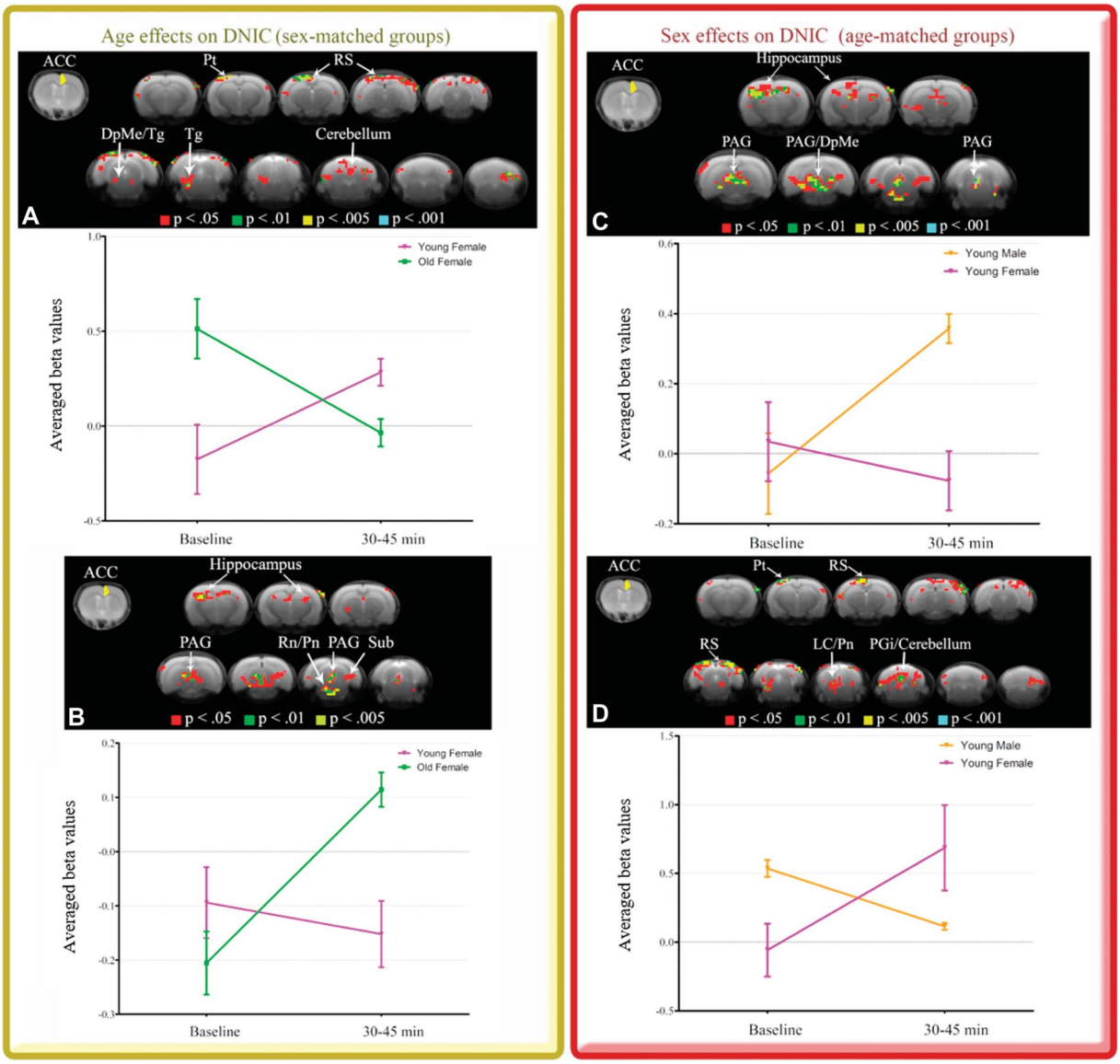

Figure 4. Right anterior cingulate cortex seed (yellow) and maps indicating unique patterns of R ACC connectivity during 30 to 45 minutes after DNIC induction. Sex-matched groups showing effects of age (A and B) and age-matched groups showing effects of sex (C and D) are illustrated. Brain images show cluster-forming thresholds at $P < 0.05$, 0.01, 0.005, and 0.001. Plots show extracted beta values from all significant clusters thresholded at $P < 0.05$ for each animal and time point (average ± SEM). DNIC, diffuse noxious inhibitory control; DpMe, deep mesencephalic nucleus; LC, locus coeruleus; PAG, periaqueductal gray; PGi, paragigantocellular nucleus; Pn, pontine nuclei; Pt, parietal association cortex; R ACC, anterior cingulate cortex; Rn, raphe nuclei; RS, retrosplenial cortex; Sub, subiculum; Tg, tegmental nucleus.

the connectivity of descending pain pathways that directly influence the endogenous ability to inhibit pain. The deficits in pain inhibition seen in old rats were associated with a brain network containing regions rich in serotonergic neurons, such as ACC, raphe nuclei PAG, and limbic areas. The blockade of serotonergic receptors in the raphe nuclei modulates the serotonin release in hippocampus and PAG, which are involved in emotional responses including anxiety.[31] The prevalence of anxiety symptoms is higher in the elderly population than in younger populations.[49] Thus, the increased connectivity between the limbic system and descending pain pathways may be associated not only with the pain inhibitory deficits, but also with the increased affective component of pain and susceptibility to comorbid mood disorders in older people.

By contrast, young rats engaged a network with heterogeneous neurochemical and functional properties during pain modulation.[9,28,38,44,45] We found that ACC and RS are the main cortical structures modulating the brain network observed in young rats. According to previous studies, RS can activate opioid and serotonergic terminals in APT that indirectly modulate responses to noxious input in the spinal cord through DpMe connections.[12,28,38,44,45] Cholinergic neurons from the Tg project to ACC and may be involved in pain modulation.[9,37,40] Indeed, we observed increased connectivity between ACC, RS, APT, Tg, and DpMe in young rats. Brain networks were also modulated by sex when we combined young and old rat: males strongly engaged PAG during DNIC, whereas females recruit a widespread network that includes ACC and RS as main cortical regions and connections to PGi, LC, and Pn. Therefore, our findings suggest that DNIC response can engage heterogeneous brain circuits to promote pain inhibition depending on age and sex.

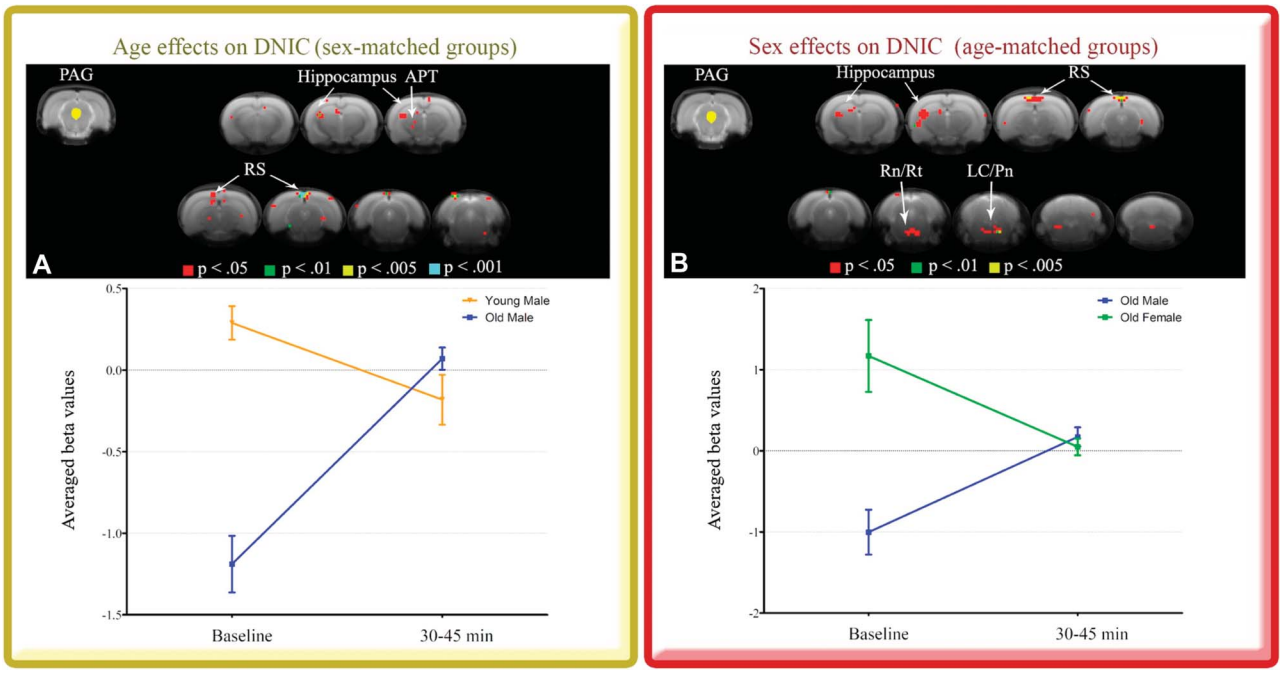

Figure 5. Periaqueductal gray seed (yellow) and maps indicating unique patterns of PAG connectivity during 30 to 45 minutes after DNIC induction. Sex-matched groups showing effects of age (A) and age-matched groups showing effects of sex (B) are illustrated. Brain images show cluster-forming thresholds at $P < 0.05$, 0.01, 0.005, and 0.001. Plots show extracted beta values from all significant clusters thresholded at $P < 0.05$ for each animal and time point (average ± SEM). APT, anterior pretectal nucleus; DNIC, diffuse noxious inhibitory control; LC, locus coeruleus; PAG, periaqueductal gray; Pn, pontine nuclei; Rn, raphe nuclei; RS, retrosplenial cortex; Rt, pontine reticular nucleus.

We advance our observations by demonstrating specific patterns of brain connectivity comparing age- and sex-matched groups. The matching procedure in research can slightly diminish the statistical power of the results.[11] However, studies with stratifications according to age and sex have elucidated how these variables can directly influence pain outcomes and

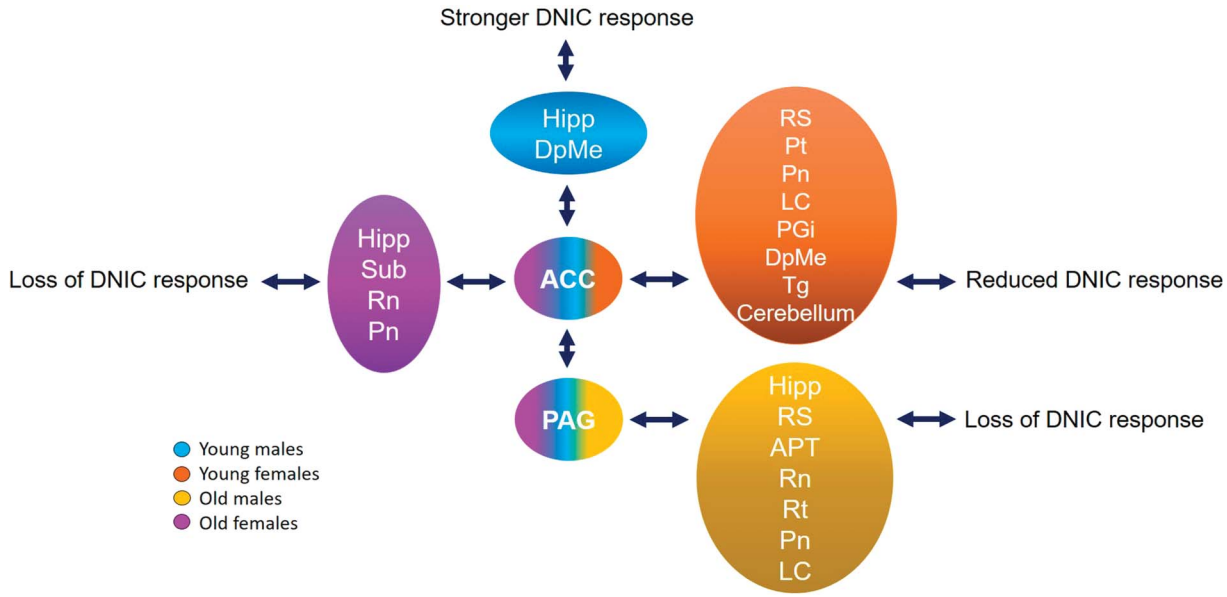

Figure 6. Model of potential pathways related to age and sex differences in DNIC. Group comparisons show brain regions that had increased connectivity during endogenous pain inhibition in rats stratified by age and sex. Anterior cingulate cortex and PAG were the ROIs (seeds), and arrows show the increased connectivity between them and several brain areas. Young males had the most efficient analgesia with the strongest connectivity between ACC and PAG (blue). The reduced efficiency of DNIC in young females seemed to be driven by a widespread brain connectivity that was not dependent on PAG (orange). Although both old groups showed behavioral loss of DNIC response, old males had increased connectivity between PAG and several brain regions that did not include ACC (yellow); and old females had increased connectivity between ACC, PAG, and more limbic regions (purple). ACC, anterior cingulate cortex; APT, anterior pretectal nucleus; DNIC, diffuse noxious inhibitory control; DpMe, deep mesencephalic nucleus; Hipp, hippocampus; LC, locus coeruleus; PGi, paragigantocellular nucleus; PAG, periaqueductal gray; Pn, pontine nuclei; Pt, parietal association cortex; Rn, raphe nuclei; RS, retrosplenial cortex; Rt, pontine reticular nucleus; Sub, subiculum; Tg, tegmental nucleus.

mechanisms.[1,25] We showed that old females have a unique pattern of brain connectivity during DNIC, which engages the limbic system to modulate the serotonergic descending pathway.[14,16,31] However, young females robustly engage multiple brain regions to achieve pain inhibition, such as noradrenergic connections between LC and ACC, RS, Pt, and PGi,[22] Pn connections to the cerebellum, which are mostly glutamate-dependent,[27] and GABAergic projections from DpMe to Tg.[37,40] Although, the increased connectivity between PAG and ACC seems to drive the most efficient pain inhibitory control seen in young males,[7] we also found that young males had increased connectivity to a limbic region (hippocampus). A point to consider is that hippocampus is divided into different regions, which have been shown to exert opposite roles in pain modulation.[48] Regarding aging, DNIC responses were lost in old males and females, but the brain networks engaged during DNIC differed in a sex-dependent manner. Old males recruited brain connections to PAG that are not ACC-dependent, whereas old females engaged ACC, PAG, and limbic regions. Diffuse noxious inhibitory control has been described as one of many components of the descending pain modulatory system, which also includes pathways associated with placebo and stress-induced analgesia.[18–20,33] We defined our behavioral and brain network outcomes as DNIC based on the widely used description of DNIC as the phenomenon of "pain inhibits pain." Descending pain pathways exert a bidirectional pain modulation, ie, inhibiting and facilitating pain.[32] Although DNIC responses were lost in both old groups, the sex differences in brain connectivity may show distinct circuities that can similarly facilitate pain. Therefore, the balance between descending inhibitory and facilitatory pathways may influence DNIC responses and this balance may be strongly modulated by sex and age.[33] We acknowledge that there were group differences in brain connectivity at baseline, whereas the behavioral responses were mostly comparable. However, the fact that each group differently engaged brain regions during the same DNIC paradigm may suggest that baseline or resting-state networks work in the same manner.

We propose a model of how potential pathways are related to age and sex differences in DNIC (**Fig. 6**). Although both old groups have shown behavioral loss of DNIC response, old females strongly have the participation of the limbic system, ACC, and PAG connections; and old males engage the limbic system and a specific PAG circuitry that are not dependent on ACC connections. The higher DNIC response observed in young males is related to increased connectivity between ACC and PAG, whereas young females have reduced DNIC responses associated with widespread connectivity that is not dependent on PAG modulation. This model also suggests potential areas for circuit manipulations to experimentally demonstrate causal relationships between brain processing and DNIC response. A potential confound of animal neuroimaging studies is the use of anesthesia, which may also be influenced by age and sex. Compared to young adult rats under long exposure (≥2 hours) to isoflurane, aged rats had altered stress responses, acetylcholine release, and hippocampal neurogenesis.[10,13] By contrast, no differences were observed in the effects of isoflurane anesthesia on behavioral responses between females and males.[23] Here, we used relatively low doses and short durations of isoflurane exposure because the brain activity and resting-state networks are generally preserved and highly reproducible between animals, compared to alpha-chloralose and awake fMRI.[6,21,47] The BOLD signal changes under isoflurane are also similar to those observed under medetomidine anesthesia.[47]

From a clinical perspective, brain mechanisms of pain inhibition are of considerable interest. Increased understanding of pain inhibitory pathways as they relate to sex and age factors can highlight potential avenues for both pharmacological and non-pharmacological interventions. Therapeutic strategies to manage chronic pain in young and elderly population are still mostly reliant on opioids, and older patients are more likely to experience cognitive impairment and fall injuries when exposed to opioids.[2,8] It is therefore feasible to speculate that if the normal aging and sex can induce maladaptive pain modulation and changes in brain circuitries, they may contribute to the low efficiency of treatment approaches to many pain syndromes and increased prevalence of chronic pain within women and older populations, which warrant further investigation.

In summary, our study provides insight into the impact of aging and sex on brain circuitries involved in pain modulation and contributes to a mechanistic understanding of the impairment in endogenous pain inhibition seen in older patients. Our findings suggest that the limbic system and widespread brain connectivity seem to enhance susceptibility to pain modulatory deficits in the elderly population, and sex may be a risk factor for developing age-related chronic pain.

Conflict of interest statement

The authors have no conflicts of interest to declare.

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Author contributions: J.T. Da Silva contributed to conception and design, acquisition of data, analysis and interpretation of data, and drafting and revising the article. C. Tricou was involved in conception and design, acquisition of data, interpretation of data, and revising the article. Y. Zhang contributed to conception and design, and acquisition and analysis of data. D.A. Seminowicz was involved in conception and design, analysis and interpretation of data, and drafting and revising the article. J.Y. Ro contributed to conception and design, analysis and interpretation of data, and drafting and revising the article.

Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/A946.

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