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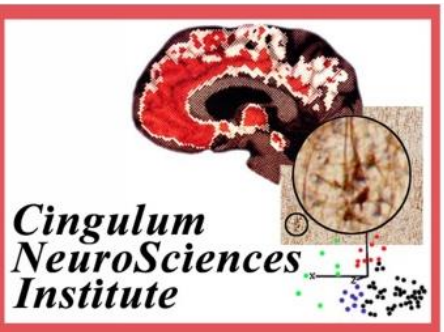

A Nociceptive-Stress Model of Adolescent Physical Abuse Induces Contextual Fear and Cingulate Nociceptive Neuroplasticities

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Abstract

Adolescent physical abuse impairs emotional development and evokes cingulate pathologies but its neuronal and circuit substrates are unknown. Conditioning adolescent rabbits with noxious colorectal distension for only 2 hours over three weeks simulated the human child abuse in amplitude, frequency, and duration. Thermal withdrawal thresholds were unchanged suggesting sensitized spinal mechanisms may not be operable. Unchanged weight, stools, colorectal histology and no evidence of abdominal pain argues against tissue injury or irritable bowel syndrome. Contextual fear was amplified as they avoided the site of their abuse. Conditioning impacted anterior cingulate and anterior midcingulate (ACC, aMCC) neuron excitability: 1) More neurons responded to cutaneous and visceral (VNox) noxious stimuli than controls engaging latent nociception (present but not manifest in controls). 2) Rear paw stimulation increased responses over forepaws with shorter onsets and longer durations, while forepaw responses were of higher amplitude. 3) There were more VNox responses with 2 excitatory phases and longer durations. 4) Some had unique three-phase excitatory responses. 5) Long duration VNox stimuli didn't inhibit neurons as in controls suggesting release of and inhibitory circuit. 6) aMCC changes in cutaneous but not visceral nociception confirmed its role in cutaneous nociception. For the first time, we report neuroplasticities that may be evoked by adolescent physical abuse and reflect psychogenic pain; i.e., no ongoing peripheral pain and altered ACC nociception. These limbic responses may be a cognitive trace of abuse and may shed light on impaired human emotional development and sexual function.

Introduction

Adolescence is a developmental period with high levels of emotion, risk-taking, and interpersonal stress that are amplified by harsh physical and sexual abuse. Sexual victimization among adolescents is estimated at 28% (Finkelhor et al., 2009) with high abuse characterized by dependency, suicidality, violence, impulsivity, substance use, and borderline tendencies (Grilo et al., 1999; Lieb et al., 2004; Fergusson et al., 2008; Pietrek et al, 2013). Adolescent physical/sexual abuse is frequent in high-psychopathy inmates (Borja and Ostrosy, 2013), childhood maltreatment increases the risk of non-specific chronic low back pain and sexual abuse enhances adult touch sensitivity (Tesarz et al., 2016). Thus, adolescent maltreatment has a significant impact over the entire lifespan.

Although child harsh abuse is influenced by socioeconomic factors (Imbierowicz and Eagle, 2003), it is initiated with intense physical pain and stress (nociceptive stress), usually by a male predator on a young female, and it is not known what occurs in the brain independent of such factors. The effort to model child abuse was inspired by a case of severe abdominal pain in a woman that was repeatedly raped as a child (Drossman et al., 2003). The physical parameters of noxious/stressful stimuli (amplitude, duration, frequency) can be simulated and neuron responses assessed immediately thereafter to show the effects of stimulation independent of human psychosocioeconomic factors; an animal model strength.

The only information available on CNS alterations following harsh physical/sexual abuse derives from imaging years later. Child abuse reduces anterior cingulate cortex (ACC) responses to colorectal distension (Ringel et al., 2008) and volume (Dannlowski et al., 2012), while in child abuse-related posttraumatic stress disorder severity correlates with anterior midcingulate cortex (aMCC) atrophy (van Harmelen et al., 2010). Although other structures may be involved (hippocampus and amygdala; see Caveats), they do not have established roles in avoidance behaviors like cingulate cortex (Gabriel et al., 1991; Vaccarino and Melzack, 1989). Also, noxious colorectal distension (nCRD) evokes ACC-dependent, conditioned place avoidance in rats (Yan et al., 2012) and it is activated by remote contextual fear (Frankland et al., 2004; Goshen et al., 2011). Thus, we employed nociceptive stress with nCRD similar to human abuse in adolescent rabbits (Methods) as human early adolescents are particularly sensitive to maltreatment (Kieley et al., 2001).

A priori hypotheses were informed by a report that ~half of ventroposterior lateral thalamic neurons had enhanced mechanical cutaneous responses following nCRD (Zhang et al., 2003) and another showing referred lumbar mechanical hypersensitivity in visceral pain (Bourdu et al., 2005). Thus, changes in visceral and cutaneous responses may be linked, neurons were evaluated for CNox-

and nCRD-evoked activity and we expected greater amplitude and duration responses after nCRD-conditioning.

For the first time, cingulate neuron nociception is shown to be altered by only ~2 hours of repeated nCRD; uncovering a latent nociceptive network, multiphase, long-duration excitation and reduced inhibition. When someone is in pain independent of peripheral stimulation, they may be experiencing this pattern of activity. These findings may provide insight into subacute chronic pain and psychiatric disorders associated with child abuse.

Methods & Materials

Derivation of the protocol. Rabbits were used because their cingulate cortex is highly cytoarchitecturally differentiated compared to rats; i.e., it has an aMCC that rat does not have (Vogt, 2015). It is about 8 times larger volumetrically than in rats and individual neurons are 3-5 times larger than in rats and this latter feature makes rabbits ideal for holding multiple neurons for long periods of stable, receptive field recording and testing.

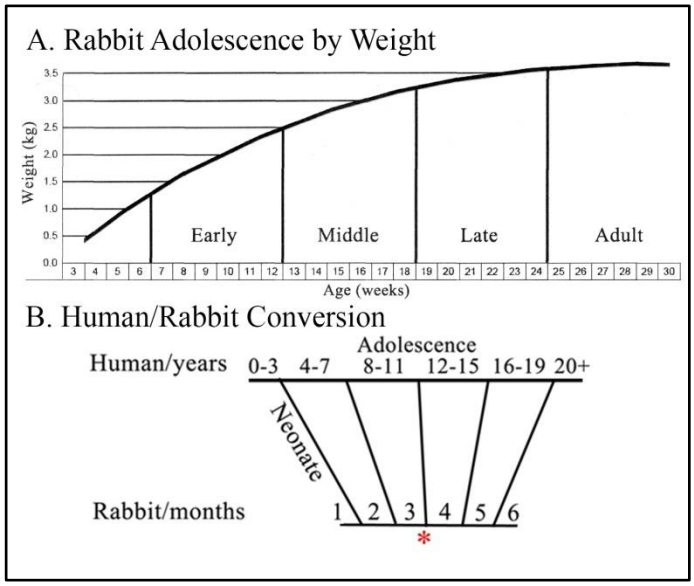
As we are attempting to model the nociceptive stress of human abuse in rabbits, it is necessary to define what constitutes human abuse to make the model parallel as close as possible human experience; i.e., enhance face validity. As noted above, nociceptive stress during predatory or disciplinary assault is at issue and not socioeconomics. *Severe physical abuse in humans is defined by being hit with an object, burning, or forced penetrative sex at least once a month for at least a year (Bremner et al., 1999).* The New York City Alliance Against Sexual Assault (web site) states, "adolescents are more likely to experience sexually violent crimes than any other age … Rape of males is any sexual assault involving forced penetration of the anus or mouth by a penis or other object." Sexual abuse is forced sex and only life-threatening force predicts adult health status (Leserman et al., 1996, 1997). Abuser gender does not predict symptomatology (Briere and Elliot, 2003) and, as children are severely affected by these events (Keiley et al., 2001), rabbits in early adolescence were used. Finally, although females are more often raped than males, males experience more profound impairments following rape (Kang et al., 2005; Vogt DS et al., 2005; Street et al., 2008). Thus, both genders were employed with the same conditioning protocol.

Adolescence in New Zealand White rabbits begins at about 11-12 weeks when the testes descend. Childhood is the time between weaning at about 4 weeks and lasts to week 12. We targeted childhood

during the 3rd-4th postnatal month which may be approximately related to human years 9-13. Figure 1 summarizes these relationships. Thus, we used nCRD because it can be employed in both genders without involving vaginal stimulation as in females only. The frequency of nCRD was determined with comparison to the human early lifespan in years compared to the rabbit early lifespan in months. For the human this is ages 8-11 for 4 years, while for the rabbit it is about the 3rd-4th months. Human harsh physical abuse is defined as at least one experience/month for at least one year (above) and involves at least 48 exposures over 4 years. As we are converting ~4 years of human life to 6 months of rabbit life, 8 or 9 nCRD exposures may equate to the human harsh physical abuse experience.

Figure 1

A. Rabbit body weight versus age in weeks. B. Conversion of human adolescence in years to that of rabbits in months. The red asterisk emphasizes the 3rd-4th month target in rabbits as childhood-early adolescence.



To summarize, the model of the physical parameters of child abuse includes 1) an age approximating late childhood/early adolescence, 2) painful stimulation (nCRD, 60 mmHg) with the distender simulating the male penis during anal intercourse, 3) short duration stimuli of ~21 min to simulate time to male ejaculation, 4) forced stimulation by holding the distender in place, and 5) repeated events (3X/week for 3 weeks). Thus, the protocol engages conscious pain systems for only a total of ~two hours.

It should be noted that the term abuse is not equivalent to nCRD. nCRD is a tool that has been used in many ways to study 1) Pavlovian conditioning over three days (Wang et al., 2000), 2) one 80 mmHg pulse for 10 mins to study oxidative stress (Vaculin et al., 2010) and 3) 12 times at 80 mmHg for drug testing (Brushberg et al., 2009) to name just a few uses of this tool. In contrast, while there are many forms of abuse, abuse in the present context is a carefully defined protocol that employs

nCRD for a specific length of time over three weeks and intensity controlled at each treatment; i.e., a level of parametric control that is impossible with human survivors.

Rabbits were housed so they could visualize and smell each other in facing cages and twice a week they were removed from their cages for play and exploration together; i.e., they were not socially isolated or neglected. Forty-four rabbits were used for these studies with some used for both behavioral and neurophysiological studies. For behavior including thermal threshold testing there were 11 controls (2.4±0.04 kg; 8 M, 3 F) and 20 nCRD-conditioned (2.5±0.05 kg; 12 M, 8 F), while for neurophysiology there were 13 controls (2.8±0.02 kg; 8 M, 5 F) and 10 nCRD-conditioned (2.4±0.03 kg; 7 M, 3 F).

All applicable international, national, and institutional guidelines for the care and use of animals were followed and this article does not contain any studies with human participants performed by any of the authors. All procedures performed in these studies received explicit and formal approval in accordance with the ethical standards of the Committee for the Humane Use of Animals at the State University of New York at Upstate Medical University and the Institutional Care and Use Committee at Northeastern University.

Behavioral testing and conditioning. Each animal received thermal withdrawal testing in a specially designed mesh sling that has a zipper at the top for rabbit insertion (Alice King Chatham Medical Arts, Hawthorne, CA). The sling keeps their body and paws elevated off the floor and allows for some movement. The sling was positioned so the animal viewed the far wall of the contextual fear testing apparatus. Water-bath, rear-leg withdrawal reflexes were used to assess the thermal threshold at temperatures between 35-54ºC. Water temperature was started at the coolest temperature and increased by 2ºC intervals with 2 min in between each. The paws are dried with a towel after the first withdrawal is evoked and a cool temperature again applied and elevated at 2ºC intervals until a withdrawal was evoked and both withdrawal temperatures averaged for the thermal threshold.

While still in the sling, nCRD conditioning was performed by first anesthetizing the animals with halothane (2%) via a mask to reduce pain as much as possible, a rectal balloon inserted as described below for sensory testing, and the anesthesia discontinued. After regaining consciousness, an session involved 7 cycles of nCRD (60 mmHg for 2 min) followed by 1 min rest. Rabbits never vocalized but their ears and tail were erect, and their respiration rate increased and was shallow. Usually on the second or third trial of the first session, the animals would arch their back and tug on their pelvic muscles to try to excrete the balloon. The CRD was held in place to prevent it from being ejected. This procedure was performed 3X/week for 3 weeks.

Following a conditioning session, the rabbit was placed in the black start box and the sling was repositioned against the far wall of the apparatus. The door of the start box was opened and the animal given 15 minutes to step down into the open chamber. The sling was 19" from the start box to assure that approaching it and palpating it required more than simply stepping out with the front paws. Control animals who had been placed in the sling for 21 mins without balloon insertion quickly stepped into the chamber, reared up on occasion and approached the sling and palpated it with their paws and noses, while nCRD conditioned animals were slow to step out of the start box and almost never approached the sling. The time to interacting with the sling was recorded and a 15 min delay without this response ended the trial. One trial was performed on days 1, 3, 5, 7, and 9.

Surgery. Following the 3 week protocol, rabbits were anesthetized with Nembutal and Xylazine (35 mg/kg and 5 mg/kg) for insertion of a trachea tube. The animal was then anesthetized for surgery with a mixture of Halothane (1.5-2%) and Oxygen (2L/min, open system). The head was fixed to the stereotaxic device by a stainless steel bar secured to the cranium with skull screws and dental acrylic. The anesthesia was then reduced to about 0.5% where only weak withdrawal reflexes could be elicited. Neuromuscular blockade was used to improve the stability of recordings with a single dose of neuromuscular blocking agent (Pancuronium bromide 2 mg/kg, i.v.), and the animal was artificially ventilated and heart rate continuously monitored during paralysis to ensure heart rate did not change during noxious stimulation. Bur holes were made in the cranium over 1-4 of the targeted areas with Anterior (A) and Lateral (L) midpoint coordinates in mm from Bregma, dorsoventral (DV; range 0, first cell activity detected to end of probe, as follows: ACC, A5, Lat 0.8, DV 1.5 to 3.5; anterior midcingulate cortex (aMCC), A1, Lat 0.8, DV 0.5-2.

Light anesthesia may influence baseline (spontaneous) activity but not nociceptive receptive fields. Studies of monkey somatosensory cortex with α-chloralose showed a minimal effect on receptive field size and no differences in the proportion of wide-dynamic range to nociceptive specific neurons (Kenshalo et al., 2000). In a study of rat ACC with α-chloralose, there was a reduction in baseline neuronal activity but it did not block nociceptive responses that were clearer than in conscious animals (Kuo wt al., 2005). This latter study showed that the same type of short- and long-latency responses to laser stimulation were evoked but that long-lasting (>4 sec) and very long latency onset (0.4 sec) responses were not present. In contrast, as shown below, we recorded very long duration cutaneous responses in ACC in nCRD-conditioned animals of 56±4 sec under light Halothane anesthesia and long onset to excitation in control animals of 22±3.2 sec. Thus, it appears that, while

there could be an alteration in baseline activity, response parameters reported in the rabbit under Halothane likely reflect those that would be recorded in conscious animals.

Sensory testing, recording and histology. Noxious percutaneous electrical stimuli (ENox) were the search stimuli as they do not damage tissue with repeated application. Subcutaneous bipolar percutaneous needle electrodes in the rear paws were used to which trains of constant current pulses (1 ms duration, 100 Hz, 50 ms; 10 sec interstimulus intervals) were applied. Mechanical cutaneous stimuli were innocuous (light brushing of the skin, gentle pressure for 5-10 s) followed by noxious cutaneous stimulation (CNox) for the same duration. CNox stimuli were strong pinches of a digit, ear or fold of skin to the point that produced a withdrawal reflex. Interstimulus intervals were 100-180 sec and cutaneous test sites were marked and spaced at least 1 cm apart. During each trial, contralateral and ipsilateral sites on the front and rear paws were tested. Finally, noxious visceral stimuli were a pressure of 60 mmHg applied for between 30 and 60 sec. The standard stimulus application trial consisted of 30 s innocuous CRD and a 3 min interstimulus interval followed by a 30s noxious CRD. All recording sites received at least 3 visceral stimulation trials, while most received 3–6 stimuli when a single level of noxious pressure was used or up to 9 when multiple levels were applied. Visceral trials usually alternated with cutaneous trials and the total time between visceral stimuli was ≥5 min.

Extracellular action potentials were recorded with two or three insulated tungsten electrodes spaced 0.5-1.0 mm apart using the Plexon multi-channel acquisition processing system (Plexon Inc, Dallas, TX). Two or three sites were recorded at different depths on each probe with at least 200 μm between sites. At the end of each penetration, small electrolytic lesions were made (10 sec, -50 nA) for localization of electrodes.

When a recording session was finished, the animals were deeply anesthetized with pentobarbital (40 mg/kg) or 6% Halothane. Following cardiac exsanguination with 0.9% saline and perfusion with 4% paraformaldehyde, brains and samples of the distal colon and rectum were removed to the same fixative for several days. Brain sections were cut at 40-μm thickness and stained with thionin for analysis of recording site locations and depths relative to electrolytic lesions. The colorectal tissues from 5 control and 7 conditioned animals were placed in 4% formalin, embedded in paraffin, cut at a 7-μm thickness and stained with haematoxylin and eosin.

Data analysis. Spike sorting and data analysis were essentially the same as that reported by Sikes et al. [55]. A two-step spike sorting approach was used to identify waveforms from single neurons (Plexon Inc., Offline Sorter software) and all sampled waveforms were aligned to their maximum

negative potentials. One to 5 clusters per electrode were identified and 3-dimensional principal component analysis was used to identify distinct waveform clusters and generate average waveform templates. Template matching was used to further refine the sorting so that the resulting waveforms were statistically significantly different in principal component space (MANOVA, $p < 0.01$), and autocorrelation interspike intervals were greater than 1.4 ms. The stability of waveform amplitude and shape was visually confirmed over the stimulation period and pairwise statistical analysis (*t*-test, $p < 0.05$) was used to select units with statistically different waveforms. The action potentials of units with statistically similar waveforms were combined unless the units had different responses to noxious cutaneous and noxious visceral stimuli.

Thirty seconds of pre-stimulus baseline activity for 3-5 stimuli was determined for each neuron against which response parameters were evaluated. Peristimulus Time Histograms (PSTH) of neuron action potential frequency with 1 sec bin widths were used to identify responses and cumulative summation analysis (NeuroExplorer Software, Nex Technologies, Littleton, MA) which shows the change in rate at each bin and bins that significantly deviate from the mean rate. Our analysis uses the following NeuroExplorer algorithm which limits the increasing standard deviation that normally occurs in Cusum graphs by using the entire histogram instead of algorithms that use only the prestimulus period to compute the graph and 95% confidence interval. Specifically, a histogram with bin counts bc[i], i=1,...,N. generates a Cumulative Sum Graph that displays the following values in the Cusum (cs[i]):

for bin 1: cs[1] = bc[1] - A

for bin 2: cs[2] = bc[1]+bc[2] - A*2

for bin 3: cs[3] = bc[1]+bc[2]+bc[3] - A*3, etc.

A is equal to the average of all bc[i] and the value of the cumulative sum for the last bin is always zero: cs[N] = bc[1]+bc[2]+...bc[N] - A*N, where A=(bc[1]+bc[2]+...bc[N])/N. The null hypothesis states there are two independent spike trains. In this case, bc[i] are independent and identically distributed (with Poisson distribution; Abeles, 1982). With an average bin count large enough (>30), a Gaussian distribution can be used. Therefore cs[i] is a random walk (at each step, it goes up or down according to a new random Gaussian value). Indeed, for the unconstrained random walk, the standard deviation of cs[n] grows with increasing n (proportional to sqrt(n)). However, in the NeuroExplorer method we used to calculate the confidence interval, the random walk is constrained: cs[N] is always zero. So all the possible trajectories of the random walk need not be considered, but only a subset of trajectories with cs[N]=0. For this subset of trajectories, the standard deviation of cs[n] is proportional to sqrt(n*(N-n)). That is, the standard deviation of cumulative sum grows initially, reaches its maximum at N/2 and then decreases. This produces an elliptical confidence interval shown in the

cumulative sum graph in NeuroExplorer showing 99% confidence limits for cs[i]. The sqrt(n*(N-n)) formula is similar to Anderson-Darling weighted statistic for the Kolmogorov-Smirnov test: https://www2.units.it/ipl/students_area/imm2/files/Numerical_Recipes.pdf formula (14.3.11).

Thus, the Cusum plots cumulative differences of PSTH bin counts from the overall mean and constructs 99% confidence intervals to identify significant changes from the mean of the entire histogram and facilitates detection of response onset/offset (Davey and Ellaway et al., 1986; Scutter and Türker, 1999; Smith et al., 2000). The onset and duration of responses were determined as deviations from the trendline (Ellaway, 1978; Oliver et al., 2001; Smith et al., 2000). A significant response to stimulation is defined as a Cusum plot where the trendline crosses the 99% CI oval ellipse for a minimum of 3 sec. Onset was the first post-stimulus bin where the trend line slope changed for ≥3 sec and offset was the first bin after response when the trendline was level or reversed direction for ≥3 sec. The time point of the beginning and end of each response Is marked by arrows in the figures. There are instances in which the 30 sec baseline period influenced determination of the onset of a response or later periods of activity. The Cusum was routinely assessed without baseline activity during the response period alone as shown for aMCC cutaneous responses in the last figure. The post-stimulus sampling period of 100-180 sec was the standard for these analyses. Amplitudes were calculated from the 3 peak bins of activity and recorded as an absolute deviation from baseline. Excitatory responses were significantly above baseline (Cusum ascending out of ellipse) and "inhibitory" responses were statistically significant below baseline (Cusum descending out of ellipse). To differentiate fluctuating neuronal discharges from the onset and duration of individual excitatory/inhibitory responses, a return to baseline or a new phase onset required an activity change lasting ≥15 sec.

Units were classified as nociceptive if they had a statistically significant change in firing during or after noxious stimulation (Cusum analysis, $p<0.01$). Chi square analysis (SPSS Crosstab analysis, $p<0.05$) was used to determine significance of response property differences with cortical regions. Effects of conditioning on response magnitude was assessed with analysis of variance (SPSS Univariate GLM analysis, $p<0.05$) with post-hoc pairwise comparisons (SPSS Bonferroni $p<0.05$). For E3 responses the non-parametric sign test was used to compare median response parameters. Data plots were made with NeuroExplorer (Nex Inc, Littleton, MA) or Matlab (Mathworks, Natick, MA).

Results

Weight and Behavioral Testing

Animals exposed to 3 weeks of the nCRD protocol had normal weight gain (initial: 1.66±0.06 kg for controls vs 1.66±0.13 kg for nCRD conditioned; after 3 weeks weights were 2.3±0.01 kg in controls vs 2.26±0.21 kg in nCRD conditioned). Stools were coded (hard-1, soft-2, fluid-3) and all animals had a normal pellet discharge (scores=1) indicating no changes in bowel habits over 3 weeks. There was no evidence for abdominal pain such as writhing, stretching, abdominal probing or hair pulling.

Thermal nociceptive testing with water baths showed no differences between controls and nCRD-conditioned animals in the threshold for withdrawal at initial testing (50.8±0.9ºC for controls vs 51.5±0.9ºC for nCRD conditioned) or after 3 weeks of nCRD conditioning (49.6±1ºC for controls vs 50.6±0.5ºC for nCRD conditioned). The withdrawal temperature before and after nCRD-conditioning on the last day of conditioning also did not change (50.3±0.5ºC). These temperatures were higher than for other species likely because rabbits have an immobility response during handling.

Conditioned animals showed anticipation of pain and stress by trying to avoid being removed from the carrying box (nose poking into bottom corners versus controls rearing to look out) and in some instances they struggled to avoid being placed in the sling. Conditioned rabbits always tried to excrete the distender by arching their backs, tail lifting, rear leg extension or walking motions, and tugging on their perineal and abdominal muscles. The head/ears and all legs were tense and fully extended and the animals often turned their heads to either side to look back. None of these nocifensive activities were observed in control animals in the sling. These findings suggest the nCRD-conditioned animals soon understood and feared the context of their abuse.

To document rabbit avoidance and fear suggested by qualitative observations, contextual fear was tested with the sling in full view of the start box. While the 5 control animals acclimated to the task over the 5 testing sessions (response times declined from 95±29 on day 1 to 17±3 sec on day 7 (p=0.04; Table 1), the 7 nCRD-conditioned animals had longer response times that did not normalize over successive testing days. Although ANOVA analyses showed no overall significant differences by group and session (p=0.056), day 7 showed a significant difference from controls (p<0.03) and trends on days 5 (p=0.08) and 9 (p=0.09). Further consideration of the 7 conditioned animals, however, showed that they did not express the same level of contextual fear; 4 of them were fearful of their environment (vulnerable), while 3 were not (resilient) with very low scores. Table 1 provides a three group analysis and there were significant differences among the group means (ANOVA p=0.005; Means±SEM:

Control 53±70, Vulnerable 437±78, Resilient 55±90). Post-hoc mean analysis showed significant differences between both the control vs. vulnerable and vulnerable vs. resilient contrasts (p=0.008 and 0.016, respectively), while that between control and resilient groups was not significant (p=0.5). Within subject analysis of the effect across days showed that within each group there was no significant change. To show the overall magnitude of differences between vulnerable and resilient animals, the 5 daily scores were summed and the difference was an order of magnitude greater for the vulnerable animals. Finally, as the behavioral and neurophysiological studies were run in parallel, most neurophysiology was done without testing and the data analysis compares controls versus all conditioned animals. Whether or not resilient animals have altered cingulate nociceptive responses remains to be determined.

Table 1: Contextual fear test (mean sec±SEM)

Day	**1**	**3**	**5**	**7**	**9**	
Control (n=5)						
	95±29	49±20	57±18	17±3#	45±25	
Conditioned (n=7)						
	250±120	315±156	223±91	264±118	316±148	
p	0.2	0.14	0.08**	0.03*	0.09**	
Three Group Analysis						
Day	**1**	**3**	**5**	**7**	**9**	
Conditioned; Contextual fear (n=4; vulnerable)						**Sum 5 days**
	404±178	533±215	349±128	355±184	545±188	2185±892
p′	0.08**	0.03*	0.03*	0.01*	0.01*	
Conditioned; No Contextual fear (n=3; resilient)						
	36±21	19±11	38±22	143±132	9±1.3	274±187
p″	0.5	0.5	0.5	0.5	0.5	
p‴	0.08**	0.04*	0.05*	0.41	0.02*	0.03*
ANOVA	0.04*	0.02*	0.02*	0.08**	0.01*	

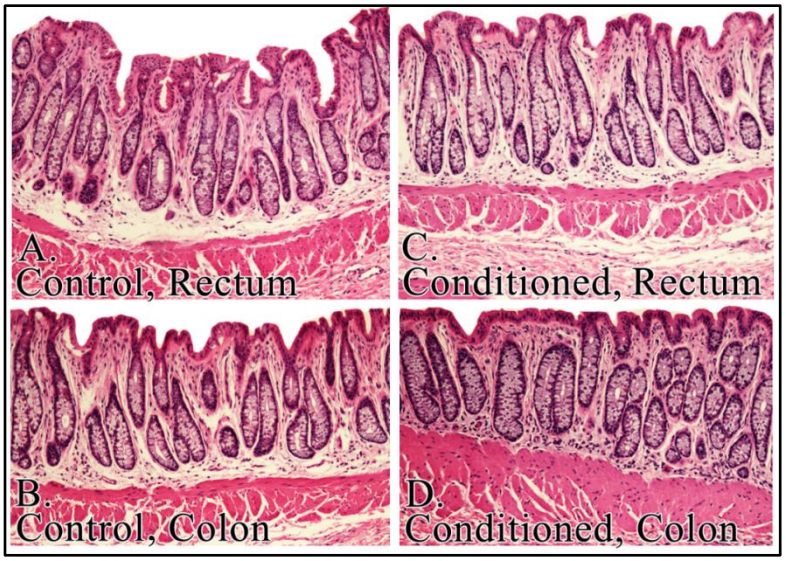
Caption:
Statistical testing: *$p\leq0.05$ significant (red); **$p\leq0.09$ trend (blue); #Control (Days vs Day 1) Paired *t*-tests. Conditioned vs. Control independent, 1-tailed *t*-tests; Three Group Analysis ANOVA (repeated measures) with Bonferroni ANOVA Overall significance, *p′* contrasts controls with vulnerable, *p″* controls with resilient, *p‴* vulnerable with resilient.

Colorectal Histology

Distal colon and rectal histology was analyzed in 4 control and 7 nCRD-conditioned animals. Figure 2 shows haematoxylin and eosin staining in control (A & B) and nCRD-conditioned animals (C & D). There was no evidence of cell damage in any layer, the glands and muscle layers were normal and there were no apparent inflammatory responses. Thus, the nCRD conditioning protocol did not produce structural damage to colorectal tissues.

Figure 2

Examples of rectal and colon histology from control (A. & B.) and nCRD conditioned (C. & D.) animals. There was no evidence of structural damage or inflammation in any layer of either structure in any conditioned animal.

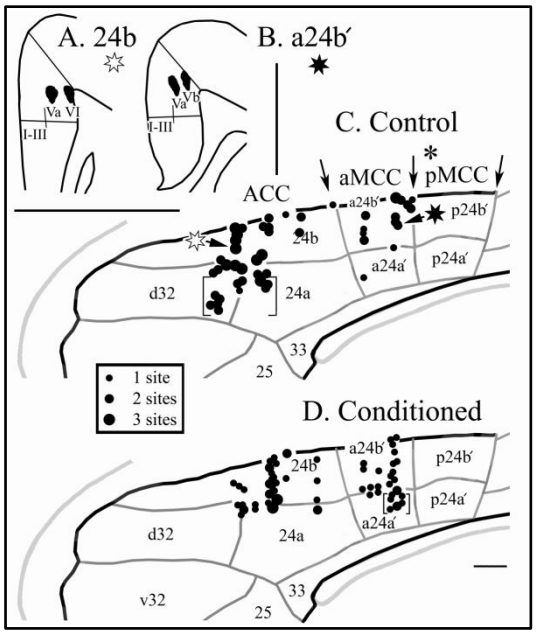


Distribution of Nociceptive Neurons

Figure 3 shows the distribution of recording sites that had nociceptive neurons in control (C.) and conditioned (D.) animals plotted onto the medial surface with areas marked according to a previous study (Vogt, 2005; asterisk designates Bregma). Examples of electrode tracks are shown in Figure 3A for layers Va and VI of area 24b (star) and in Figure 3B for layers Va and Vb of area a24b′ (solid star). While most sites in control ACC were in areas 24b and d32, there were 22 sites with a ventral placement that were not present in conditioned animals (Fig. 3C. brackets). Statistical comparisons between CNox for these 29 neurons versus the 81 dorsally placed neurons for forepaws and for rear paws 32 ventral versus 73 dorsal neurons showed no differences in responses and were combined for data analysis. The VNox responses were all the same except for the onset and duration of E2 and Table 2 presents these values for the dorsally placed neurons only. Also, there were 6 sites representing 16 neurons in conditioned aMCC with a ventral location (brackets, Fig. 3D.) and these did not differ from

the 32 neurons with a dorsal placement for all measures except for the onset of the first excitatory phase (E1) to rear CNox. Except for the latter, the two groups were combined for analysis (Table 3).

Figure 3



A. Pairs of electrode tracks in a control case with nociceptive neurons in layers Va and VI of area 24b and B. layers Va and Vb of area a24b′. Active sites are noted with a star and filled star, respectively in C. Control distribution of recording sites. Bracketed sites were compared with all other dorsal sites and no differences were identified in response variables resulting in combining all neurons from all sites for statistical analyses. Asterisk is the position of Bregma. D. Distribution of recording sites with nociceptive neurons in Conditioned animals. The brackets identify ventral sites that were compared to dorsal ones and all data were the same except for onset of E1 from which they were excluded in Table 3. Scale bar, 1 mm.

The number of neurons tested per animal was assessed. Neuron numbers for ACC CNox did not differ between groups: control, 14.8±3.8 (N=13) vs conditioned 15.6±2.4 (N=8). For VNox controls the number of neurons per animal were 19.7±4.9 (N=9) vs conditioned 16.9±3.4 (N=7). For aMCC the CNox values for neurons/animal for controls were 16.9±3.5 (N=7) versus conditioned 23.3±3.4 (N=6), while for VNox controls they were 12.7±3.2 (N=7) versus conditioned of 14.4±3.5 (N=5). Thus, no animal contributed disproportionately to the findings as roughly the same numbers of neurons were tested/animal.

Sensory Responses

Nociceptive responses: ACC

Table 2 summarizes key ACC data including the number of neurons tested by group (control/conditioned), stimulus type (CNox/VNox) and response valence (excited/ inhibited). ACC neurons were more frequently nociceptive in nCRD-conditioned than control animals; only 15% and 11% were not responsive (NR) to CNox and VNox, respectively, in conditioned animals, while in controls 30% and 31% were NR. The overall frequency distribution for responsive and NR neurons in control and conditioned animals was highly significant (p=0.004) and the frequency distributions by response class for controls vs conditioned animals were also highly significant for VNox (p=0.0001) but not CNox.

Table 2: ACC

CNox		Control		Conditioned	
Total tested		193		124	= 317
Resp/% Group		136/70%		105/85%*	
NR/% Group		57/30%		19/15% (overall, 0.004**)	
Responsive neurons		Front	Rear	Front	Rear
Number/%		110/81%	106/78%	90/86%	88/84%
Mean Sum Dur Es		39±3	43±3	47±3	56±4 (0.01)
I N		3	7	8	11
E N		84	72	52	39
IE N		2	6	3	8
EI N		11	12	13	18
EE N		9	12	16	14
E1: N/%		105/52%	96/48%	80/54%	69/46%
Onset		22±3.2	24±3.6	16±2.4	13.6±2.9 (0.03)
Duration		37±2.9	38±3.1	39±3.2	47±5
Amplitude		2.6±0.2	3±0.2	3.4±0.2(.007)	3.5±0.3
E2: N/%		21/19%	24/23%	29/32%	30/34%
Onset		71±11	73±10	64±6	68±6
Duration		35±8	43±7.1	37±4.2	44±6.3
Amplitude		4.3±1.2	2.7±0.5	3.2±0.5	3.1±0.5
VNox		Control		Conditioned	
Total Neurons		181/60%		118/40%	= 299
Resp/% Group		89/49%		84/71%	
NR/% Group		92/51%		34/29% (overall, 0.0001)	
E Responsive					
N/Stim Duration<50s		64/30±0.1		24/34±0.4	
N/Stim Duration≥50s		23/60±0.4		44/62±0.5	
N/Sum Es Dur<50s		64/48±4.8		24/52±5	
N/Sum Es Dur≥50s		23/44±5.6		44/62±0.5	
All stimuli r		0.49		0.17	
E N		64		42	
EI/IE N		8		8	
EE N		8		20	
EEE N		1		7	
I N		8		7 (overall, 0.013)	
Onset	E1	76/36±4		71/32±4	
	E2	13/62±6		27/82±7 §§	
	E3	1/55		8/77±13 (0.027***)	
Duration	E1	76/44±4		71/47±4	
	E2	5/51±19		27/30±3 §§	
	E3	1/58		8/30±9	
Amplitude	E1	76/2.7±0.2		71/2.8±0.2	
	E2	5/2.4±0.4		27/3.1±0.4	
	E3	1/6.1		8/1.6±0.2 (0.008***)	

*chi-square test significant at $p<0.05$ for all frequency distributions; p values in parentheses ≤0.03
**overall refers to the total frequency distribution) and *t* tests for differences between means±SEM Bonforroni corrected
red highlighted pairs are values associated with $p<0.05$
§§ Control ventral ACC neurons in brackets in Figure 3C removed for final calculations of E2 onset and duration for comparison with conditioned neurons.
***Sign test for significance
Abbreviations: Dur, duration; Es, excitatory responses; I, inhibitory; N, number; NR, no response; r, correlation coefficient; Resp, Responsive neurons; Stim Dur, VNox stimulus duration

Cutaneous nociception. The nociceptive stress protocol evoked significant alterations in cutaneous responses. There was a clear enhancement of CNox excitation in conditioned animals as shown by the greater percent of responsive neurons, and shorter onset and longer duration of the first excitatory (E1) response. A shift in cutaneous receptive field organization was evidenced by higher amplitude front paw responses in conditioned animals, an increased frequency of EE responses to front paw stimulation and a significant enhancement of summed excitatory response duration in the rear versus front paws.

Responses either formed single excitatory or inhibitory responses or there were multiple excitatory phases distinguished by either a return to baseline or inhibition for ≥15 sec. Summation of all excitatory responses (Table 2; "Mean Sum Dur Es") measures overall excitability and shows that while there was no difference in the front paws; conditioned neurons had longer durations than rear paws (56±4 vs 43±3 sec). The frequency distribution according to response type (I, E, IE, EI, EE) did not differ between front and rear paws in either group. However, the first excitatory phase (E1) in E, EE, and EI responses differentiated controls from conditioned animals, although EE did not. The E1 phase in conditioned animals had a significantly earlier onset to rear paw stimulation than controls (13.6±2.9 vs 24±3.6 sec) and greater amplitude to forepaw stimulation (3.4±0.2 vs 2.6±0.2 spikes/sec).

Figure 4 shows examples of the patterns of rear paw responses in controls. The excitatory responses can be categorized as short (Fig. 4A.), medium (Fig. 4B. & C.) or long (Fig. 4D.) duration. Examples of second phase responses are also shown with short duration excitatory followed by inhibitory responses (Fig. 4E.) or medium duration excitatory responses followed by inhibition (Fig. 4F.). Figure 4 provides examples of the patterns of cutaneous, rear paw responses in conditioned animals classified as for controls. Besides the short, medium and long excitatory responses and subsequent inhibitory responses, novel primary inhibitory (Fig. 4H.) and inhibitory followed by excitatory responses are apparent (Fig. 4I. & J.).

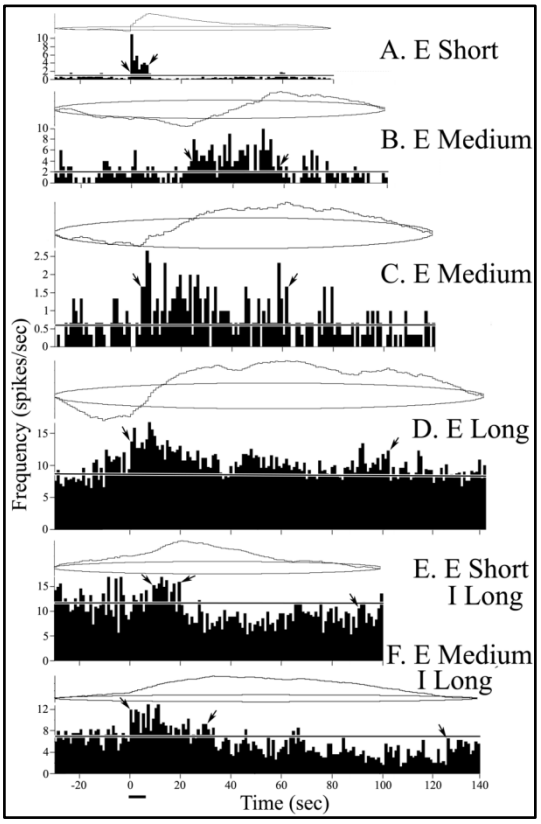


Figure. 4

ACC neuron responses to rear paw CNox stimulation in control animals; Cusum above each histogram shows the 99% confidence interval, the baseline is indicated with horizontal lines and stimulus duration is shown with the bar at the time scale. Arrows mark the beginning and end of each response determined from Cusum and baselines. Examples of a full range of response durations are shown for excitatory (E) responses. A. Short, B. and C. Medium and D. Long. Secondary inhibitory responses are shown in E. and F.

Examples of rear paw responses in conditioned animals are shown in Fig. 5. Generally speaking, the patterns of cutaneous responses were similar to those in controls; however, they are affected by selection biases and durations were longer and there were more complex responses than in controls. These differences are best evaluated in Table 2.

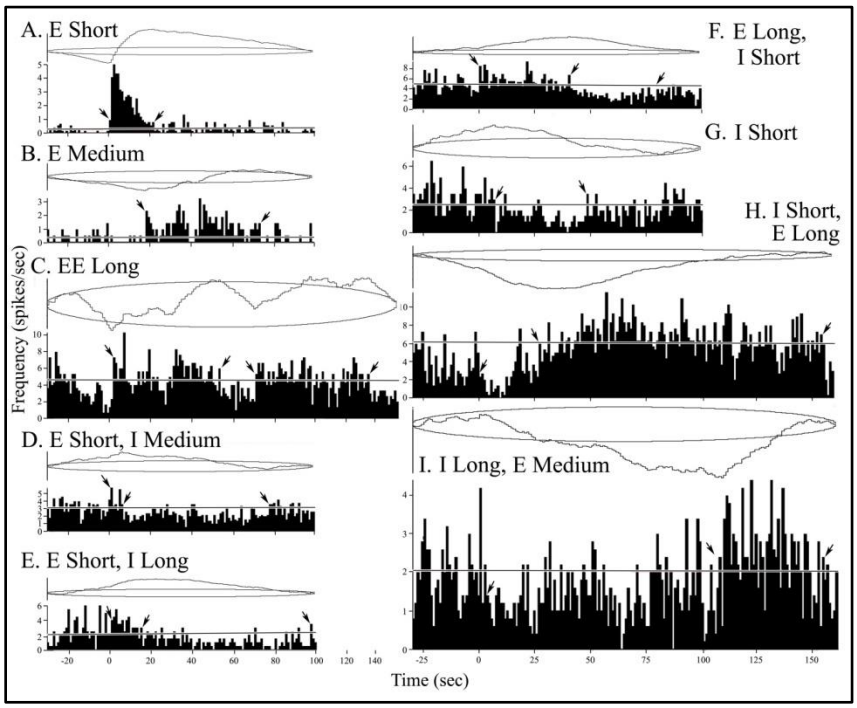


Figure 5
Examples of ACC neuron responses to rear paw CNox stimulation in conditioned animals. A. and B. Short- and medium-duration excitation. While inhibition followed excitation (D., E., F.), there was evidence of multiple excitatory (C. EE) and primary inhibitory responses (H., I., J.). Some of these latter responses were followed by excitatory activity (I., J.).

Visceral nociception. VNox responses were more frequent in conditioned than control animals and controls appeared to have an inhibitory shaping of excitatory responses to long-duration VNox stimuli not present in conditioned animals. The amplitude of E2 responses was greater and there were numerous E3 responses in conditioned animals not in controls. While an inhibitory phase often occurred in the latter neurons, these responses were often followed by another excitation not seen in controls. Finally, the overall pattern of excitation in controls was graded and uniform, while in conditioned animals the presence of intermediate inhibitory and multiple excitatory phases suggests a more precise coding of visceral stimuli.

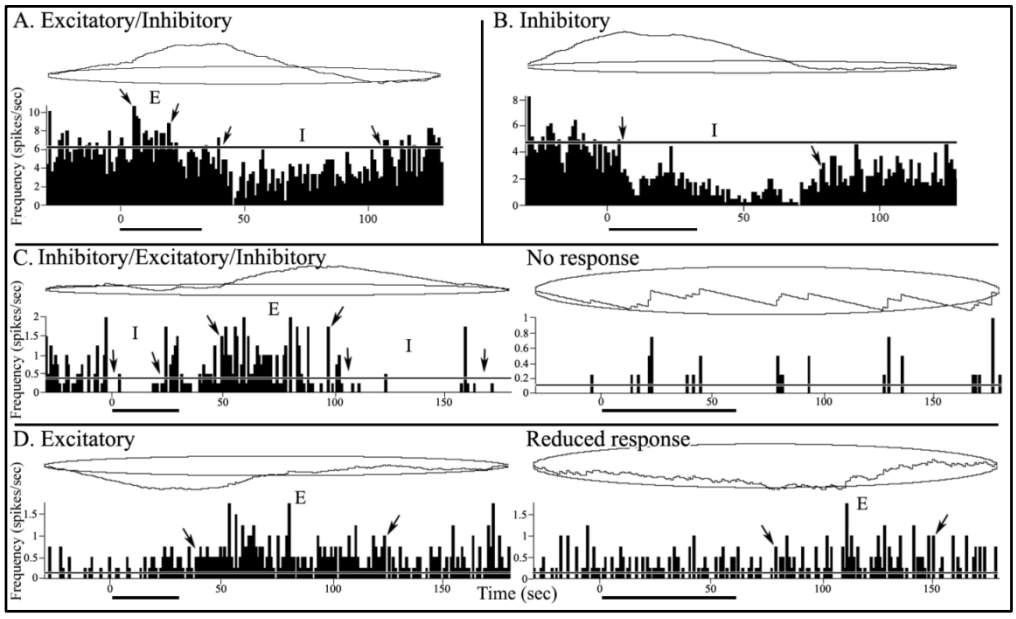
There were significantly more VNox-responsive neurons in conditioned (71%) than control (49%) groups (Table 2; $p<0.001$). The VNox stimuli varied in duration from 30-65 sec and more control neurons were tested with shorter (<50 sec, N=64) than longer (≥50 sec, N=24) stimuli. In spite of this, the summed excitatory responses were similar for both groups at short-duration stimuli, while those in the conditioned animals were longer than controls (62±0.5 vs 44±5.6 sec) and this difference can be explained by differences in stimulus duration. Counter intuitively, however, longer duration stimuli in controls were often not related to longer duration responses suggesting the latter evoke an inhibitory mechanism. This phenomenon was evaluated in two controls where 13 units evoked excitatory

responses to 30 or 60 sec duration stimuli. Five of these units converted from excitatory at 30 sec to NR at 60 sec duration stimuli and some excitatory responses were reduced in amplitude.

The average pattern of excitatory responses in controls with subsequent inhibitory activity is shown in Figure 6A and an instance of pure inhibition in Figure 6B. Two further examples of control responses demonstrate the effect of stimulus duration and the likely presence of an inhibitory mechanism not present in conditioned animals. Figure 6C shows a neuron that was excited by 30 sec, 60 mmHg stimuli but was NR to the 60 sec stimuli. In a second example, the excitatory response evoked by 30 sec stimuli was greatly reduced and delayed during 60 sec stimuli (Fig. 6D.). Also, the sum of excitatory responses actually shortened, though not significantly, from 71±19 to 42±14 sec. Finally, there was no correlation between stimulus duration and summed excitatory responses for all neurons tested in control (r=0.49) or conditioned (r=0.17) animals.

Figure 6

ACC responses to VNox stimuli in 4 control neurons. A. and B. evoked E/I and I-only responses, respectively. The unique property of control neurons was the frequent reduction in activity with long-duration stimuli. C. I/E/I response to 30 sec stimuli converted to NR at 60 sec. D. A 120 sec-duration excitatory response at 30 sec reduced to a 70 sec response by 60 sec stimuli.



The frequency distribution by response type was very significantly altered by conditioning (Table 2; p=0.0013). The highest percentage of control neurons responded with a single excitation (72%) versus only 50% in conditioned animals. The critical outcome of conditioning was to generate complex patterns of VNox-evoked responses with EE (23%) and EEE (8%). There was only one in controls.

The excitatory pattern of responses in conditioned animals was more complex than in controls and the presence of E3 responses and double the number of E2 responses demonstrates the unique coding properties of VNox stimuli in conditioned animals. The onset and amplitude of E1 responses were similar for both groups; however, E2 onset was longer (82±7 vs 62±6 sec) and duration shorter (30±3 vs 51±19 sec) in conditioned animals. Also, there was only one E3 responses in controls but 8 in conditioned animals.

Conditioned neurons coded VNox stimuli differently than controls. Figure 7 shows examples of VNox responses in conditioned animals with each phase marked. The presence of three phases did not depend on nCRD stimulus durations as they were detected following 30 sec (C., 30 mmHg) and 60 sec (B., 60 mmHg) duration stimuli. Of particular note is the response in Figure 7B.; at 30 mmHg it evoked a single, medium duration excitatory response, while at 60 mmHg it evoked very robust onset and offset discharges. This pattern was never observed in control neurons. The neuron in Figure 7D emphasizes another pattern in which the 30 mmHg stimulus evoked a very long excitatory burst, while another neuron (Fig. 6E) evoked a long-duration inhibitory response at 60 mmHg.

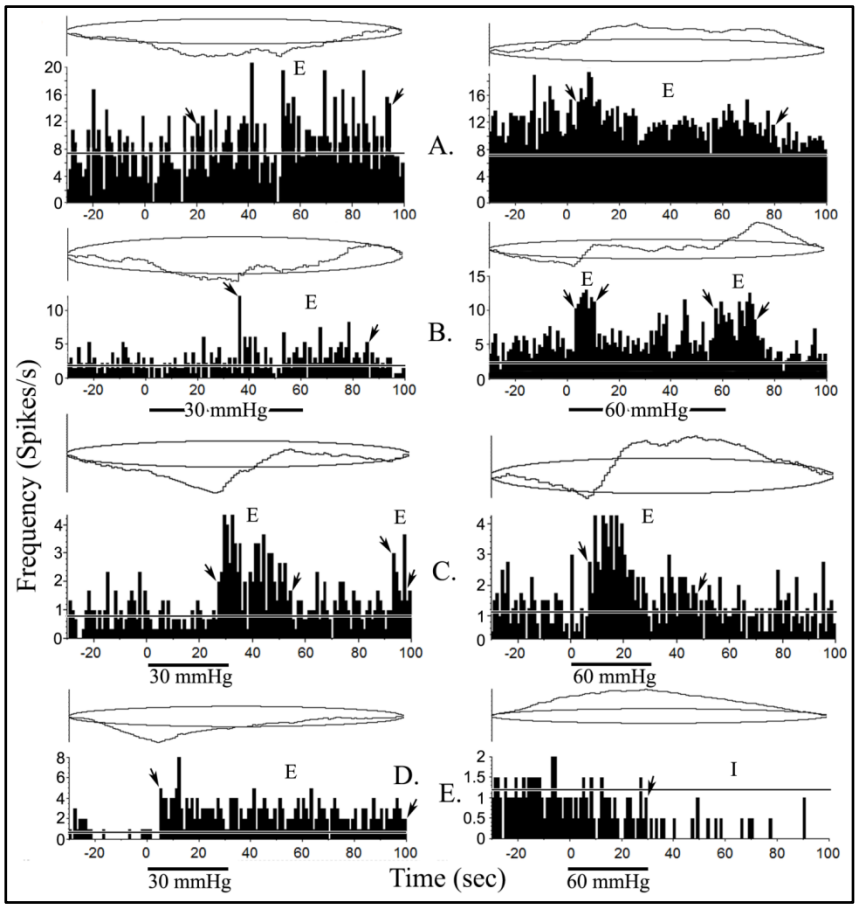


Figure 7
ACC responses to VNox stimuli in conditioned animals at 30 and 60 mmHg on the left and right, respectively. In two instances the responses were essentially the same (A., C.), while in one the response was amplified by 60 mmHg stimulation (B.) and in another E converted to I when 30 sec stimuli were used (E.). The 60 mmHg response in B. showed clear links to onset and offset which leads to the conclusion that additional phases in conditioned animals more effectively coded visceral stimulation parameters.

Nociceptive responses: aMCC

Cutaneous nociception. Conditioning in aMCC uncovered a latent cutaneous nociceptive system (one that is present but not expressed), excitatory responses were lengthened and there was a loss of forepaw inhibited-only neurons. Table 3 summarizes neuron responses in aMCC. While there were significantly more conditioned neurons responsive to CNox as in ACC, the proportion of VNox-responsive neurons did not change emphasizing a primary role in cutaneous sensation for aMCC. Summation of the durations of all excitatory phases showed that substantial lengthening occurred in the conditioned animals (front, p=0.007; rear, p=0.03). Also, there were 8 neurons with only inhibitory responses to FCNox in controls but none in conditioned animals, although a similar number were present for RCNox. There were 3-phase responses to stimulation of either paw but none in controls. Finally, chi square for all response classes differed significantly between groups (p=0.01).

Table 3: aMCC

CNox		Control		Conditioned	
Total tested		87		82	= 169
Resp/%		59/35%		70/41%*	
NR/%		28/17%		12/7% (overall, 0.007**)	
Responsive neurons		Front	Rear	Front	Rear
Number		42	44	53	59
Mean Sum Dur Es		38±6	53±5	57±4 (0.007)	69±5 (0.03)
I	N	8	6	0	5
E	N	26	23	31	30
IE	N	0	5	2	5
EI	N	2	5	6	4
EE	N	6	4	8	10
3 phase	N	0	0	6	5 (overall, 0.01)
E1:	N	34	32	50	48
	Onset	19±5	26±6	24±4	7±2§ (0.002)
	Duration	35±6	49±6	48±5	56±6
	Amplitude	3.3±0.4	2.8±0.3	3.4±0.3	4.3±0.6
E2:	N	6	9	12	17
	Onset	94±15	65±12	76±12	56±6
	Duration	19±6	42±9	35±4	46±7
	Amplitude	1.8±0.2	3.0±0.4	3.7±0.5	3.4±0.9
E3:	N	0	0	5	4
	Onset			94±9	88±16
	Duration			48±14	65±16
	Amplitude			5.0±2.5	6.1±2.7
VNox		Control		Conditioned	
Total Neurons		89		72	= 161
Resp/% Total		47/29%		45/28%	= 92
NR/% Total		42/26%		27/16% (overall, 0.14)	
Stimulus Duration					
N/Stim Duration<50s		69/30±0.1		30/32±0.2 (0.001)	
N/Stim Duration≥50s		20/59±2		42/62±2 (0.001)	
N/Sum Es Dur<50s		31/41±7		17/75±7 (0.002)	
N/Sum Es Dur≥50s		8/49±10		26/68±8	
Stimulus x E Dur		r=0.08		r=-0.10	
E	N	28		28	
EI	N	2		3	
EE	N	5		8	
EEE	N	1		1	
EIE	N	0		1	
I	N	8		2	
IE	N	3		4	
IEE	N	0		1	
Total	N	47		45 (overall, p=0.34)	
Onset	E1	36/35±6.2		39/41±5.9	
	E2	9/76±11.5		12/69±8.8	
Duration	E1	36/37±5.8		39/65±5.7	
	E2	8/38±13.1		12/44±5.4	
Amplitude	E1	36/2.7±0.4		39/3.3±0.5	
	E2	9/3.1±1		12/3.1±0.3	

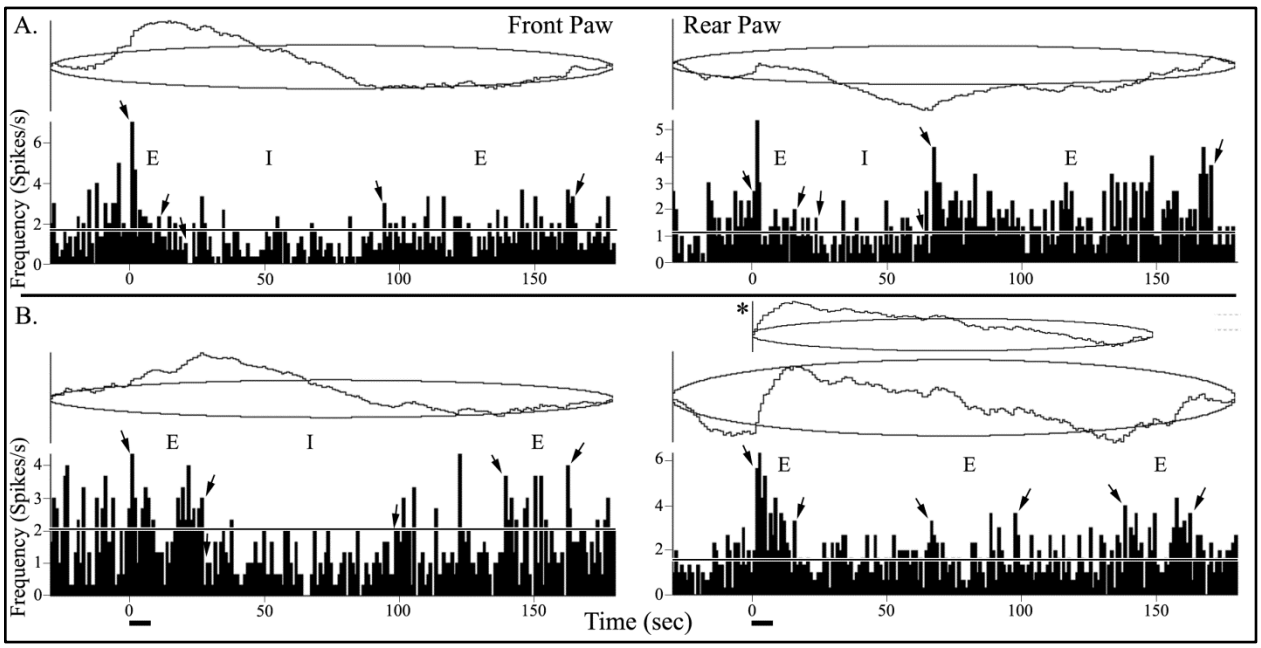
*chi-square test significant at p<0.05 for all frequency distributions
**overall refers to the total frequency distribution)
t tests for differences between means±SEM; red highlighted pairs are values associated with p<0.05 Bonforroni corrected
*p values in parentheses≤0.03.
§Mean does not include the ventrally located neurons in aMCC conditioned animals with different onset than dorsal neurons.
Abbreviations: Dur, duration; Es, excitatory responses; I, inhibitory; N, number; NR, no response; r, correlation coefficient; Resp, Responsive neurons; Stim Dur, VNox stimulus duration

Response parameters for the three excitatory phases were also modulated by conditioning; E1 had very short onset in the rear paws (7±2 vs 26±6 sec) and a higher amplitude, while there were no differences in E1 durations. In contrast, E2 onset and amplitude were similar for both groups but their duration was almost twice that of controls (35±4 vs 19±6 sec). Finally, there were no E3 responses in controls but there were for both

front and rear paws in conditioned animals. Figure 8 shows the complex CNox responses in aMCC. These include an EIE evoked from both paws and in another neuron the EIE response in the forepaw was associated with an EEE response upon stimulation of the rear paw.

Figure 8

CNox responses in aMCC for two neurons. A. EIE evoked from both paws. B. EIE response in the forepaw was associated with an EEE response upon stimulation of the rear paw. Asterisk, insert: The Cusum is essentially a moving average related to the 99% confidence integral. Although all figures show the pre-response baseline period, we routinely checked the Cusum without the 30 sec pre-stimulus response to assure that the initial response was statistically significant. In this instance the Cusum is shown for the 150 sec response period.



Visceral nociception. VNox responses were essentially unchanged by conditioning; a very different profile from ACC. Chi square analyses of the frequency distributions for VNox-responsive neurons were not significant for total responsive/NR neurons or by response classification. As was the case for ACC, stimulus durations in control and conditioned groups differed with shorter ones in the controls and longer ones in the conditioned animals. While the duration of VNox stimuli were longer in conditioned animals and much which was accounted for by stimulus duration, there was no correlation between stimulus duration and the total duration of excitatory responses (control r=0.08; conditioned r=-0.1).

Discussion

These are the first experiments to evaluate a nociceptive-stress model similar to child physical abuse and a neurobiology of these events is possible where only human imaging was available. The nCRD protocol did not alter colorectal histology (no tissue damage), thermal withdrawal thresholds (no sensitization) or stools and abdominal pain (not irritable bowel syndrome). However, (1) contextual fear was elevated. (2) Increased frequency of nociceptive neurons in ACC indicated recruitment of a nascent nociceptive circuit that may enhance attention to painful stimuli. (3) Enhanced ACC excitability over controls suggested release of an inhibitory brake; >duration of rear paw excitation, onset of E1 half of controls, and >amplitude of forepaw E1, while VNox evoked two times more EE responses with durations >two times controls and a unique EEE pattern. (4) aMCC responses differed from ACC as VNox-responsive neuron frequency was unchanged but CNox responses were lengthened and 3-phase responses appeared. Thus, cutaneous and visceral nociceptive coding are altered by the nCRD protocol in cingulate cortex and may provide a cognitive signature of abusive events that enhance nociceptive stress and avoidance behaviors.

Contextual fear and dissociation of ACC from spinal reflexes

All rabbits did not express the same level of fear suggesting vulnerable/resilient animals as known in stress research (Kalisch et al., 2015; Kleim et al., 2015). Ackerman et al. (1998) showed that ~30% of children exposed to physical/sexual abuse developed PTSD and Kasai et al. (2008) reported these groups in combat exposed twins with or without PTSD. Yehuda and Antelman (1993) suggested that an appropriate stress model should not always elicit PTSD-like symptoms. In terms of pain, Walsh et al. (2007) reported that 24% of physically/ sexually abused children had no change in pain responses but 36% experienced pain that interfered with some or most daily activities.

Lack of enhanced thermal withdrawal further suggests this is not chronic pain and we are unaware of any study that used only 2 hours of noxious stimulation over 3 weeks to generate spinal sensitization that would impact ACC function. Furthermore, dissociation of spinal reflexes and ACC-mediated nociception/pain is documented with ACC lesions; lesions do not alter spinal nociceptive excitability (Pastoriza et al., 1996), reduce formalin-induced conditioned place avoidance without reducing pain reflexes (Johansen et al., 2001) and decrease bee venom-evoked paw lifting but not paw flinching (Ren at al., 2008). Finally, anticipation of pain modulated ACC neuronal activity but not that in mediodorsal thalamus or somatosensory cortex (Wang et al., 2008).

The nociceptive-stress model is not chronic visceral pain

This conclusion is based on the following: 1) Thermal withdrawal thresholds were the same for both groups in contrast to rat visceral pain models (Messaoudi et al., 1999; Al-Chaer et al., 2000; Bourdu et al., 2005). 2) Our rabbits showed no signs of abdominal pain (arched posture, abdominal fur pulling or palpation, body stretching). 3) Visceral pain models use continuous, conscious noxious stimulation (~500 hours/3 weeks) and show spinal sensitization, while our animals received only 2 hours of pain and the conscious pain burden for chronic models is ~250X that of our animals. 4) The E2/E3 responses are not observed in chronic visceral pain. Instead, CRD during rat inflammatory visceral pain evoke generalized increases in ACC discharges beyond 5 min interstimulus intervals, excitatory responses are high during stimulation, the same pattern occurs to all stimuli, and hyperexcitability occurs at a lower level poststimulation (Gao et al., 2006). 5) ACC cutaneous responses in rat are unchanged by chronic pain (Gao et al., 2006), while nCRD-conditioned animals had an increased number of CNox neurons and aMCC E3 responses. 6) All animals exposed to chronic pain remain therein; e.g., all exposed to spinal nerve ligation experience pain for >6 months (Decorsterd and Woolf, 2000). In contrast, animals resilient to the nCRD protocol suggests some selective vulnerability. Thus, in the absence of spinal sensitization and low total peripheral pain, we may have isolated an anticipatory mechanism that is maintained cognitively by altered limbic circuitry.

Psychogenic pain or limbogenic nociceptive stress

Pain reports may not have a close relationship to peripheral noxious stimulation and Nakamura et al. (2014) introduced the term “psychogenic pain” which we use to refer to physiochemical changes in ACC/aMCC and other areas engaged in fear (below). Feeling pain in the absence of noxious stimuli or not reporting it following peripheral trauma reflects activity in salience networks (Borsook et al., 2013) including ACC/aMCC (Seeley et al., 2007). Further, centralized pain syndromes like fibromyalgia absent robust peripheral pathology and intervention responses (Clauw, 2014) are limbic mediated and child abuse/rape are vulnerability factors for it (Imbierowicz and Egle, 2003; McBeth et al., 1999). While cingulate cortex is often implicated in pain, its role in top-down, avoidance control is poorly understood and cingulate reorganization in the nociceptive-stress model may explain these phenomena.

MCC plasticity

The aMCC is most frequently activated in humans by acute noxious cutaneous stimuli (Vogt, 2005). Since rodents do not have an aMCC like lagomorphs (Vogt 2015), this and a previous study (Sikes et al., 2008) provide the first neuronal information on this subregion. The latter study showed that aMCC has proportionately fewer VNox-only neurons and more CNox-only responders. VNox responses in Table 3 show these were essentially unchanged by conditioning in aMCC, while those for CNox neurons were; confirming this hypothesis.

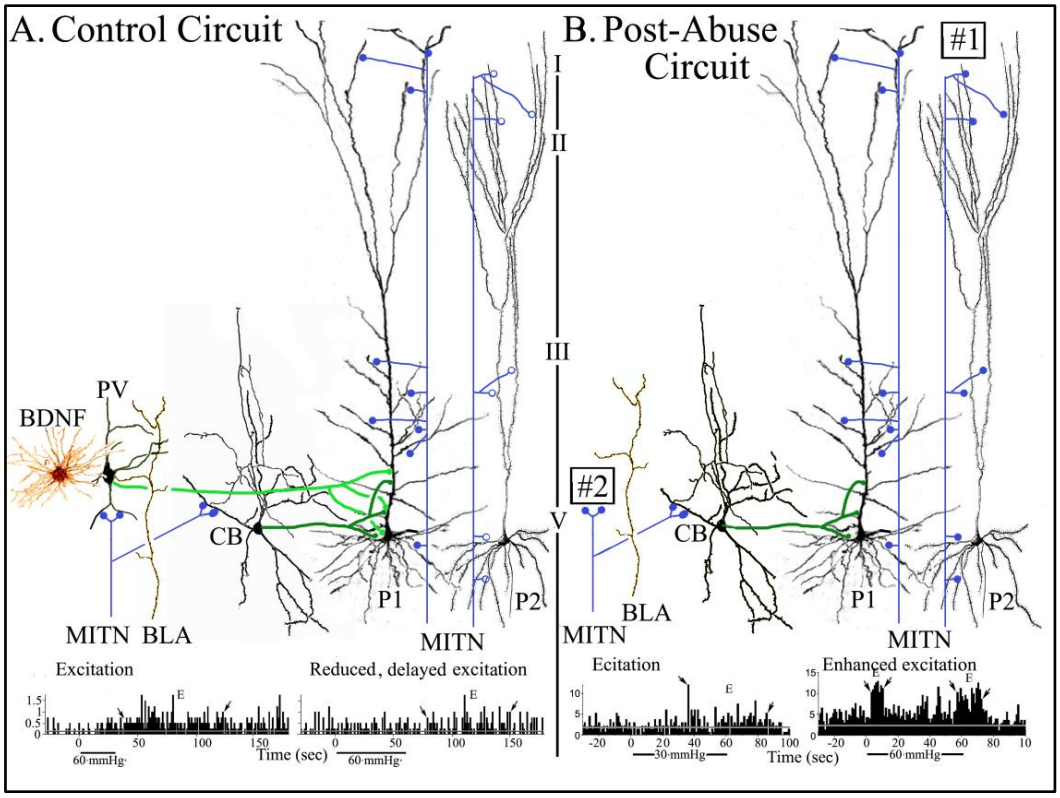
Circuit model of key findings

The two key changes evoked by the nCRD protocol are diagrammed in Figure 9. The robust increase in the proportion of CNox and VNox responsive neurons (#1 in Fig. 9B) suggests a previously unknown neuroplasticity. This is likely not due to axonal sprouting of nociceptive thalamic afferents but rather may reflect activation of previously inactive thalamocortical synapses as is true for activation and sensitization of silent C nociceptors (Handwerker et al., 1991).

An inhibitory circuit in controls was uncovered by long-duration VNox stimuli that was removed by conditioning. Figure 9B (#2) shows a source of this brake may be the basolateral amygdala (BLA) which terminates on parvalbumin (PV)-expressing inhibitory neurons in ACC (Gabbott et al., 2006) and BLA inhibits ACC neurons (Ji et al., 2010; Dilgen et al., 2013). Since dendritic growth in PV+ amacrine cells depends on Brain-Derived Neurotrophic Factor (BDNF; Rickman, 1999) released mainly from glia, conditioning may interfere with BDNF production and <PV in ACC to impair inhibitory but not excitatory transmission (Sakata et al., 2009). Thus, inhibitory control could be released by nCRD conditioning by reducing or removing PV expression and its BDNF support.

Figure 9

Pictoral summary of key findings and possible changes in ACC-associated circuitry evoked by the nociceptive-stress protocol with emphasis on layer V that receives the heaviest input from BLA (Bacon et al., 1996). **A. Control Circuit.** This is composed of 5 cells: astrocytes that express BDNF, an intrinsic PV neuron that depends on BDNF support and receives BLA afferents (modified from Gabbott et al., 2006; axonal projection in light green and BLA afferent in yellow), a multipolar neuron that may express calbindin (CB; Vogt and Peters, 1981; intrinsic projection added in dark green), a nociceptive pyramid (P1; Shyu et al., 2010), and a Golgi-impregnated pyramid (P2, Vogt and Peters, 1981). The midline, mediodorsal and intralaminar thalamic nuclei (MITN) projections are in blue. Active terminals are filled circles to P1 and inactive terminals, implying this neuron is not nociceptive, are empty (P2). The two histograms are from a unit in Fig. 5 and show that lengthened VNox stimuli evoke reduced and delayed responses implying an inhibitory intervention in the response. **B. Post-Nociceptive/stress Circuit.** Two key features appear to be evoked by this protocol; #1 reflects more active MITN synapses (silent synapses uncovered) that result in more neurons being activated by noxious stimuli (P2). #2 indicates that BDNF expression is reduced and loss of support of the PV neuron removes it from the circuit. There is not a complete loss of inhibition as suggested by activity of the "CB" neuron. The histograms are from Fig. 6 and show a moderate excitatory response to innocuous stimuli that may be greatly enhanced by noxious stimulation. This particular nociceptive response suggests that the nociceptive-stress circuit results in a more effective coding of the onset and offset of VNox stimulation.



Implications for disease and human experience

As this model may represent psychogenic or limbic-centralized pain, it is possible rewiring in subacute pain is due thereto. Survivors of childhood abuse report significantly more chronic pain than those without such histories (Davis et al., 2005) and ~33% of adult women with chronic spine pain had two or more childhood traumas including physical/sexual abuse (Ciccone et al., 1997). Also, adults reporting childhood chronic pain are more likely to report a history of physical/sexual abuse (Hassett et al., 2013). Thus, psychogenic-nociception/fear may be a vulnerability factor for adult-onset chronic pain.

Increases in the proportion of CNox- and VNox-responsive neurons may explain why somatization, including diffuse pain symptoms, frequently follow child abuse/rape and abuse severity correlates with adult somatic complaints. Sphincter of Oddi patients have excessive non-gastroenterological somatic complaints and child sexual abuse is high (Abraham et al., 1997). Also, a history of abuse increases vulnerability to emotional distress and a tendency to attend, amplify, and over-interpret somatic symptoms (Riley et al., 1998; catastrophizing).

Child physical abuse/rape are associated with increased risks of later sexual disorders including vulvodynia (Harlow and Stewart, 2005) and stress and urge incontinence (Davila et al., 2003). Finally, 60-94% of women surviving sexual abuse report sexual impairments (Leonard and Follette, 2002) and fear of sexual contact. Terr (1991) documented a girl that was sexually abused between ages 5-15. At 38 she feared sex with her husband unless she initiated it and she avoided positions taken by her father as they stimulated fear, pain and revulsion. This latter response is one of contextual fear and may be mediated by impairment of cingulate function.

Caveats and assumptions

The suggestion that the nCRD protocol is one of abuse refers to the animal's treatment and not internal perceptions that are unknown, although the animal's behavior indicates physical abuse during nCRD in the sling. All mammals can be abused with physical maltreatment, injury, assault/beating, violation or neglect. It is *not* assumed, however, that the animal protocol is equivalent to human abuse as unique psychosocioeconomic factors modulate the latter. It is also not assumed that all forms of abuse (e.g., CNox vs VNox) evoke the same CNS changes and this needs further experimentation. Indeed, the human cingulate cortex is profoundly more elaborate than that of the rabbit, although the latter does have an aMCC not present in rodents. Thus, the animal abuse model cannot exactly replicate that of the human experience. Finally, it is not assumed that only adolescents can be abused; child abuse is simply more severe than in adults (Keiley et al., 2001).

The phrase "simulated the physical parameters of the primary events of human child abuse" reflects nCRD amplitude (noxious), duration and frequency similar to the physical abuse of females (Methods; Derivation of Model; Bremner et al., 1999). Also, “primary” refers to the precipitating physical events not the class of abuse, internal perception, or secondary socioeconomic modulators; although social variables can be analyzed in animal models with maternal neglect and social groupings. Thus, we do not imply this model equates to human physical abuse/rape as no animal model could. However, it is suggested the nCRD protocol is a “human-relevant” form of child physical abuse.

Animal models have met with varying degrees of success which is measured by predictive outcomes; i.e., drug trials and other treatments in human patients. In the case of Alzheimer’s disease, there have been no efficacious therapeutics to result from rodent models including transgenic animals (Drummond and Wisniewski, 2017). However, there are many instances in which human pathology can be informed by animal studies. For example, the baboon appears to be useful in stem-cell based therapies for Parkinson’s disease with optimization for graft localization and volume (Grow et al., 2017). The common marmoset has promise for developing immunotherapies for multiple sclerosis as it has a human-like immune system and pathological grey matter lesions (Kap et al., 2016). Of particular relevance to the present study is the fact that animal studies can inform us of the various environmental and genetic mechanisms through which postnatal challenges can produce long-term effects on brain-behavior relationships; i.e., pathways through which early stress might produce long-term effects and point to systems that moderate risks for psychopathology in humans (Driscoll and Barr, 2016). Finally, in terms of experimental animal pain studies, there are a few examples in which translation to the human clinic have failed like the failure of neurokinin-1 antagonists such as MK-869 to produce analgesia (Hill, 2000). However, these few failures must be weighed against the extensive animal models that have provided a wealth of information on nociceptive neuron properties, pain pathways in multiple species and neurotransmitter function and dysfunction in multiple models of chronic pain (Mogil et al., 2010). The bottom line is that no experimental animal replicates the human condition including the present one, but extensive information can be gleaned from them regarding specific brain-behavior mechanisms that are not possible to achieve in humans.

Finally, cingulate cortex is unlikely to be the only limbic region engaged by this protocol. The amygdala is hyperactivated when humans are exposed to subliminal, negatively valenced pictures (Dannlowski et al., 2013) and is involved in contextual fear in rats (Angele-Figuera et al., 2005). Indeed, there is a network engaged in contextual fear that is likely relevant to nociceptive stress including the hippocampus, amygdala and ACC (Maren et al., 2013). Finally, an overall perspective

on contextual memory and responding is provided by the concept of emotional embodiment (Niedenthal, 2011). This concept ties together the perceptual, combinatorial and motoric reeperiencing of embodied emotions in specific networks that could be critical to contextual fear via a partial reactivation of the experience.

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