Repeated exposures of naïve and neuropathic pain-suffering mice to serpents in an experimental model to study post-traumatic stress disorder

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Abstract

Confrontation of rodents by natural predators provides a number of advantages as a model for traumatic or stressful experience. Using this approach, one of the aims of this study was to investigate a model for the study of post-traumatic stress disorder (PTSD)-related behaviour in mice. Moreover, because PTSD can facilitate the establishment of chronic pain (CP), and in the same way, patients with CP have an increased tendency to develop PTSD when exposed to a traumatic event, our second aim was to analyse whether this comorbidity can be verified in the new paradigm. C57BL/6 male mice underwent chronic constriction injury of the sciatic nerve (CCI), a model of neuropathic CP, or not (sham groups) and were submitted to different threatening situations. Threatened mice exhibited enhanced defensive behaviours, as well as significantly enhanced risk assessment and escape behaviours during context reexposure. Previous snake exposure reduced open-arm time in the elevated plus-maze test, suggesting an increase in anxiety levels. Sham mice showed fear-induced antinociception immediately after a second exposure to the snake, but 1 week later, they exhibited allodynia, suggesting that multiple exposures to the snake led to increased nociceptive responses. Moreover, after reexposure to the aversive environment, allodynia was maintained. CCI alone produced intense allodynia, which was unaltered by exposure to either the snake stimuli or reexposure to the experimental context. Together, these results specifically parallel the behavioural symptoms of PTSD, suggesting that the snake/exuvia/reexposure procedure may constitute a useful animal model to study PTSD.

Keywords: chronic pain; post-traumatic stress disorder; defensive reaction; pain modulation; prey versus serpents confrontation paradigm; *Epicrates cenchria crassus*.

Abbreviations: post-traumatic stress disorder (PTSD); chronic pain (CP); chronic constriction injury of the sciatic nerve (CCI); elevated plus-maze (EPM).

1. INTRODUCTION

Wild venomous (Coimbra et al., 2017a; Calvo et al., 2919a,b) and non-venomous (Guimarães-Costa et al., 2007; Lobão-Soares et al., 2008; dos Anjos-Garcia et al.,

2019) snakes are increasingly used as threat stimuli in studies of defensive behaviours, with a particular goal of analysing the potential relationships between exposure to these predators and animal models of anxiety disorders (Coimbra et al., 2017b).

These studies follow a long history of research using confrontations of laboratory rats and mice by natural predators such as *Felis silvestris catus* to elicit defensive responses and enable their measurement under varying circumstances (e.g. Blanchard and Blanchard, 1989; Ribeiro-Barbosa et al., 2005). Notably, cat odour alone can elicit a strong defensive response (Apfelbach et al., 2005; Dielenberg and McGregor, 2001; Takahashi et al., 2005) and can be used as an unconditioned stimulus for rapid fear conditioning (Dielenberg and McGregor, 2001; Hubbard et al., 2004). This observation suggests that predators can be used to provide both unconditioned and conditioned threat stimuli, combinable in ways that potentially extend the range of paradigms designed to produce behaviours that are similar to symptoms of various psychopathologies. In fact, snake exposure has been suggested (Coimbra et al., 2017a; Guimarães-Costa et al., 2007; Lobão-Soares et al., 2008) as an experimental model of panic attack and used to test novel and established drugs with panicolytic-like effects (Coimbra et al., 2017b; Paschoalin-Maurin et al., 2018; Twardowschy et al., 2013; Uribe-Mariño et al., 2012), as well as neuromodulators and the neural networks underlying the control of defensive behaviour (Almada and Coimbra, 2015; Almada et al., 2015).

The goal of this study was to determine the effects, in mice, of multiple exposures to a snake and to stimuli associated with the snake. In this context, an advantage of using snakes as predator threats is that they shed their skin periodically, producing exuviae, layers of shed skin. These exuviae provide both snake odour and a visual stimulus somewhat similar to that of the snake itself, potentially eliciting some degree of unconditioned defensiveness, an issue evaluated here in Experiment 1. Simultaneous exposure to both a snake and its exuvia should further increase responsivity to the exuvia, enhancing defensiveness to a stimulus that already serves as an unconditioned threat. Thus, Experiment 2 utilises extended snake exposure, over 4 h, followed at intervals by simultaneous exposure to snake and exuvia and later by exposure to the exuvia alone, with appropriate measures to determine how this protocol may alter the expression of individual defensive behaviours.

The hypothesis of the present work was that mice threatened by a natural predator, a wild constrictor snake, and reexposed to the aversive context and partial snake cues would show exacerbated anxiety/fear-related defensive reactions, some of which were similar to those displayed by post-traumatic stress disorder (PTSD) patients, potentially providing support for mouse-snake confrontation as a new model to study PTSD. Since PTSD could facilitate the establishment of chronic pain (CP) (Sharp and Harvey, 2001; Villano et al., 2007), and in the same way, patients with CP have an increased tendency to develop PTSD when exposed to a traumatic event (Gibson, 2012), we also hypothesised that chronic neuropathic pain would enhance defensive responses to the snake and to a conditioned aversive context. The interactive effects of chronic pain with snake plus conditioned aversive context exposure was evaluated by examining allodynia and behaviours displayed by prey in the elevated plus-maze test (EPM), a test of anxiety-like behaviour.

2. RESULTS

2.1. Results of Experiment 1

The *Epicrates cenchria crassus* snakes were kept in the open area of the enclosure, and explored the polygonal arena for snakes versus prey confrontation. Although it could reach the elevated platforms for escape, they demonstrated a place-preference for the enclosure floor, waiting the approach of their potential prey. Even being previously fed, they still reacted vigorously when prey were close to them, threatening their potential prey. In this case, either offensive (with attempt to bite) or defensive (without bites) strikes were observed, however, no mice were actually harmed by the snakes.

Risk assessment. Mice exposed either to the exuvia or to the snake exhibited a higher frequency and duration of risk assessment (Bonferroni's *post hoc* test, $p < 0.05$) when compared to non-threatened (control) animals. Moreover, animals exposed to the snake also showed a higher duration of risk assessment than those exposed to the exuvia (Figure 1A and B). [One-way ANOVA indicated significant effects on frequency ($F_{2,20} = 21$, $p < 0.0001$) and duration ($F_{1,20} = 20$, $p < 0.0001$) of risk assessment.]

Defensive immobility/freezing. Mice exposed to the snake exhibited a higher frequency and duration of defensive immobility/freezing (Bonferroni's *post hoc* test, $p < 0.05$) when compared to non-threatened animals or to those exposed to the exuvia (Figure 1C and D). [One-way ANOVA showed significant effects on frequency ($F_{2,20} = 7.4$, $p < 0.01$) and duration ($F_{1,20} = 8.3$, $p < 0.01$) of defensive immobility.]

Time in protected areas. Mice exposed to the snake spent more time in the protected areas (Bonferroni's *post hoc* test, $p < 0.05$) when compared to non-threatened animals or to those exposed to the exuvia (Figure 1E). [One-way ANOVA showed significant effect on time spent in protected areas ($F_{1,20} = 14$, $p < 0.001$).]

Escape. Mice exposed either to the exuvia or to the snake showed a higher frequency of escape behaviour (Bonferroni's *post hoc* test, $p < 0.05$) when compared to non-threatened animals (Figure 1F). [One-way ANOVA indicated a significant effect on the frequency of escapes ($F_{2,20} = 23$, $p < 0.0001$).]

2.2. Results of Experiment 2

Threatened mice showed both unconditioned and conditioned fear-induced defensive responses, and there were no significant effects of nerve injury on the frequency or duration of risk assessment (Figure 2A and B), frequency or duration of defensive immobility (Figure 2C and D), time in protected areas (Figure 2E) or escape behaviour (Figure 2F).

Risk assessment. Both sham and CCI mice exposed to the snake exhibited a higher frequency and duration of risk assessment (Bonferroni's *post hoc* test, $p < 0.0001$) than animals not exposed to the predator. During reexposure to the experimental context, animals previously exposed to the snake displayed a decrease in the frequency and duration of risk assessment than they exhibited during exposure to the predator. However, it is important to highlight that animals previously exposed to the snake continued to exhibit a higher frequency of risk assessment (Bonferroni's *post hoc* test, $p < 0.0001$) than those never exposed to the predator (Figure 2A and B). There were significant effects of the following factors: presence of the snake (three-way ANOVA, risk assessment frequency: $F_{1,62} = 188.22$, $p < 0.0001$; duration: $F_{1,62} = 98.48$, $p < 0.0001$), experimental context (risk assessment frequency: $F_{1,62} = 11.06$, p

< 0.01; duration: $F_{1,62}$ = 15,53, p < 0.001) and the interaction of the snake exposure and experimental context (i.e. only mice exposed to the snake showed high frequency and duration of risk assessment during exposure to the experimental context: risk assessment frequency: $F_{1,62}$ = 39.45, p < 0.0001; duration: $F_{1,62}$ = 42.98, p < 0.0001).

Defensive immobility/freezing. Both Sham and CCI animals exposed to the snake exhibited a higher frequency and duration of defensive immobility than those not exposed to the predator (Bonferroni's *post hoc* test, p < 0.0001). During reexposure to the experimental context, animals previously exposed to the snake showed reduced frequency and duration of defensive immobility (Bonferroni's *post hoc* test, p < 0.001) than they exhibited during exposure to the predator (Figure 2C and D). There were significant effects of exposure to the snake (three-way ANOVA, defensive immobility frequency: $F_{1,62}$ = 22.11, p < 0.0001; duration: $F_{1,62}$ = 12.31, p < 0.001), exposure to the experimental context (defensive immobility frequency: $F_{1,62}$ = 15.03, p < 0.001; duration: $F_{1,62}$ = 10.09, p < 0.01) and the interaction of snake exposure and experimental context, i.e. only mice exposed to the snake showed high frequency and duration of defensive immobility during exposure to the experimental context: Defensive immobility frequency: $F_{1,62}$ = 14.12, p < 0.001, duration: $F_{1,62}$ = 9.45, p = 0.01).

Time in protected areas. During the reexposure to the experimental context, animals that were previously exposed to the snake exhibited a decrease in the time spent in the protected areas (Bonferroni's *post hoc* test, p < 0.001) when compared to that shown during exposure to the experimental context (Figure 2E). There were significant effects of exposure to the snake (three-way ANOVA, $F_{1,62}$ = 33.02, p < 0.0001) and an interaction of snake exposure and experimental context (i.e. only mice exposed to the snake showed much time in the protected areas during exposure to the experimental context: $F_{1,62}$ = 19.75, p = 0.0001) on time spent in protected areas. Both sham and CCI animals exposed to the snake spent more time in the protected areas than those not exposed to the snake (Bonferroni's *post hoc* test, p < 0.001).

Escape. Furthermore, during the reexposure to the experimental context with the snake exuvia, a similar panic attack-like response was elicited (Figure 2F). There was a significant effect of exposure to the snake (three-way ANOVA, $F_{1,62}$ = 189.08, p < 0.0001) on the frequency of escape. Both sham and CCI animals exposed to the

snake exhibited a higher frequency of escape than those that were not threatened by the predator (Bonferroni's *post hoc* test, $p < 0.0001$).

Grooming. Both Sham and CCI mice exposed to the snake exhibited a higher frequency of grooming than those not exposed to the predator (Bonferroni's *post hoc* test, $p < 0.05$). This high frequency of grooming was maintained during the reexposure to the experimental context. CCI but not sham mice showed higher grooming durations during exposure than their non-threatened controls (Bonferroni's *post hoc* test, $p = 0.01$). This difference was not significant during reexposure to the experimental context (Figure 3A and B). There were significant effects of exposure to the snake (three-way ANOVA, frequency: $F_{1,62} = 6.55$, $p < 0.05$; duration: $F_{1,62} = 22.77$, $p < 0.0001$), as well as an interaction of context with nerve injury (duration: $F_{1,62} = 8.17$, $p < 0.05$) and an interaction of exposure to the snake, experimental context, and nerve injury (i.e. only during the snake exposure, CCI threated mice showed much longer time of grooming when compared to the CCI non-threated group: $F_{1,62} = 5.27$, $p < 0.05$).

Rearing. Sham and CCI animals exposed to the snake exhibited a lower frequency and duration of rearing than non-threatened mice during exposure (Bonferroni's *post hoc* test, $p \leq 0.05$) but not reexposure to the context (Figure 3C and D). There were significant effects of exposure to the snake (three-way ANOVA, frequency: $F_{1,62} = 16.27$, $p < 0.001$; duration: $F_{1,62} = 3.92$, $p = 0.05$) and of the experimental context (rearing frequency: $F_{1,62} = 10.57$, $p \leq 0.01$; duration: $F_{1,62} = 5.18$, $p < 0.05$).

Crossings in the polygonal arena. Snake-exposed Sham and CCI animals made fewer crossings than non-threatened rodents during exposure (Bonferroni's *post hoc* test, $p < 0.0001$), but not during reexposure to context only. During reexposure, mice previously exposed to the predator made more crossings (Bonferroni's *post hoc* test, $p < 0.01$) than they had done while exposed to the snake (Figure 3E). The effect of snake exposure on crossings was significant (three-way ANOVA, $F_{1,62} = 30.39$, $p < 0.0001$), as was the effect of experimental context ($F_{1,62} = 7.58$, $p \leq 0.01$) and the interaction of exposure to snake and experimental context (i.e. only mice exposed to snakes showed fewer crossings during exposure to the experimental context: $F_{1,62} = 6.38$, $p < 0.05$).

Ten days after surgery, CCI mice showed a higher nociceptive response, i.e., allodynia, when compared to themselves prior to surgery and to sham animals after surgery [two-way ANOVA; effects of the nerve injury procedure (baseline vs. 10 days after surgery; $F_{1,66} = 33.78$, $p < 0.0001$, Bonferroni's *post hoc* test, $p < 0.001$), sham vs. CCI interaction ($F_{1,66} = 6.04$, $p < 0.05$, Figure 4)]. Importantly, at that point, the animals had not yet been exposed to the snake; therefore, they were only divided into two groups according to CCI surgery: sham and CCI.

Considering nociceptive behaviour (evaluated by von Frey test), the effects of threat and aversive contextual exposure, the sham group exposed to the snake showed a decreased withdrawal response to mechanical stimulation (i.e., antinociception) recorded immediately after the exposure to the predator and from 15 to 45 min later. On reexposure to the aversive contextual environment with snake exuvia, there were significant effects of nerve injury (three-way repeated measures $F_{1,31} = 46.14$, $p < 0.0001$), snake exposure ($F_{1,31} = 38.88$, $p < 0.0001$) and an interaction between injury and snake exposure ($F_{1,31} = 41.95$, $p < 0.0001$). This last statistical effect means that, interestingly, snake-exposed sham animals displayed allodynia 6 days after the last exposure, (i.e., before reexposure), and this hypersensitivity to mechanical stimuli persisted after reexposure to the aversive contextual environment (Bonferroni's *post hoc* test, $p < 0.0001$). Mice with neuropathic pain displayed a long-lasting allodynia in both situations, i.e., before and after exposure to the predator (Bonferroni's *post hoc* test, $p < 0.0001$) and before and after (Bonferroni's *post hoc* test, $p < 0.0001$) reexposure to the experimental context (Figure 5). During exposure, there were significant effects of nerve injury (three-way repeated measures MANOVA, $F_{1,31} = 72.69$, $p < 0.0001$), snake exposure ($F_{1,31} = 14.89$, $p \leq 0.001$) and time ($F_{5,155} = 9.70$, $p < 0.0001$). There were significant interactions between the following factors: nerve injury and snake exposure ($F_{1,31} = 13.89$, $p \leq 0.001$), nerve injury and time ($F_{5,155} = 10.37$, $p < 0.0001$), snake and time ($F_{5,155} = 10.41$, $p < 0.0001$), and among all three interventions (interaction among snake, nerve injury and time, i.e. Snake-exposed CCI mice showed allodynia when compared to Snake-exposed Sham mice immediately after the exposure to the predator and from 15 to 45 min later: $F_{5,155} = 11.08$, $p < 0.0001$).

EPM. Snake-exposed mice spent significantly less time on the open arms than mice not exposed to the snake (two-way ANOVA, $F_{1,31} = 4.84$, $p < 0.05$; Bonferroni's

post hoc test, $p < 0.05$). No other measures (frequencies of entries into the open and closed arms) showed significant changes to either snake exposure or CCI or the interaction between them (Figure 6).

3. DISCUSSION

In the present studies, repeated exposure of mice to a constrictor snake or its exuvia induced defensive responses related to fear, e.g., defensive immobility, escape, and increased time spent in protected areas, and to anxiety, such as risk assessment (Blanchard et al., 1993; Coimbra et al., 2017a; Graeff, 1994; Gray and McNaughton, 2000; McNaughton, 2011). Such results corroborate our previous data, in which Swiss or C57BL/6 mice, gerbils and hamsters were confronted by a constrictor or venomous snake (Almada and Coimbra, 2015; Almada et al., 2015; Coimbra et al., 2017a,b; Guimarães-Costa et al., 2007; Lobão-Soares et al., 2008; Twardowschy et al., 2013; Uribe-Mariño et al., 2012) and displayed a range of defensive responses. In addition, study 1 indicated that the exuvia, alone and without previous association with the snake, elicited some defence behaviour, albeit at a lower level than that which was observed (study 2) when the exuvia had previously been encountered along with the snake. The odour of a predator *per se* is well established to be able to cause aversion (Apfelbach et al., 2005; Dielenberg and McGregor, 2001; Takahashi et al., 2005). Comparisons between the effects of, for example, cat odour (Mackenzie et al., 2010; Souza and Carobrez, 2016) and snake odour (Dell'Omo and Allevia, 1994; Carere et al., 1999), in addition to the present effects seen with exuvia, suggest that the latter include a rather modest unconditioned response (study 1), that appears to be enhanced when the exuvia have been encountered together with the snake (study 2). These data, and the enhanced defence behaviours seen in response to the polygonal arena without the snake after the threatening exposure (performed in that same polygonal arena), demonstrate that the snake can serve both as unconditioned stimuli, for defensive conditioning to the exposure context, and to enhance defensiveness to a cue stimulus (the exuvia) that normally elicits a low but significant level of defensiveness.

These conditioning effects were somewhat behaviourally specific, largely involving increased numbers of risk assessment behaviours and escape behaviours

rather than defensive immobility (freezing). The latter is perhaps unsurprising given that (1) several days of pre-exposure/habituation to the context had been given before threat exposure; (2) mice may not exhibit as much freezing behaviour as laboratory rats (Blanchard et al., 2001); (3) we used a protocol of conditioned fear different from those commonly used in the literature, in which the unconditioned fear stimuli, usually foot shocks, are paired with neutral visual and olfactory clues and/or with the experimental context (aversive environment) where they were presented (Curzon et al., 2009); (4) the large size of the arena (140 x 62 x 50 cm), coupled with the presence of routes of escape and avoidance, such as the stairs/elevated platforms and burrow, may have permitted the animal the selection of other more efficient defensive behaviours, such as risk assessment and flight behaviour (Blanchard et al., 1989; McNaughton and Corr, 2004).

Regarding risk assessment, Blanchard et al. (2011) noted that the information obtained through this behaviour is extremely important in determining the most appropriate defensive behaviour, such as freezing, if the animal cannot flight, or escape, when there are safe places for the animal to hide. Importantly, risk assessment behaviour seems to play a fundamental role in both mild and intense stress situations since it facilitates the acquisition of information about the threat stimulus and situation, leading to the intensification of defensive reactions if the aversive stimulus is identified or to the reduction of those reactions if the threat is not found (Blanchard et al., 1997).

Both during the exposure to the polygonal arena or in the reexposure to the experimental context, mice exposed to the snake presented a higher frequency of grooming than those not threatened by the predator. Although grooming is one of the most frequently observed motor activities in mice (Fentress, 1988; Reeves et al., 2016), it can be even more frequently exhibited when these animals are exposed to some types of stressful situations (Kalueff et al., 2016), leading some authors to consider it a displacement behaviour (Cohen and Price, 1979).

Although the CCI procedure produced consistent effects on the von Frey test, indicating hypersensitivity to mechanical stimuli, it had very little impact on any of the measures of responsivity to the snake, nor did it alter behaviour on the EPM test. While this lack of change in defensive responses or anxiety was unexpected in view of a range of previous findings suggesting that the sciatic nerve ligature procedure may

decrease open-arm proportions in the EPM test and other anxiety measures (e.g. Narita et al., 2006; Zhang et al., 2014), the ligature procedure used here was less damaging than is often employed (e.g. Zhang et al., 2014), potentially suggesting that a threshold for the effect of pain on anxiety and defence behaviour may be involved. Moreover, it is important to highlight that some other studies corroborate our results showing that neuropathic pain conditions do not evoke anxiety-like behaviours in mice, evaluated by EPM exposure and other different behavioural tests, 3 and 84 days following spared nerve injury (SNI) surgery (Pitzer et al., 2019) and 7, 14 and 28 days after partial sciatic nerve ligature (PNL) (Hasnie et al., 2007). Also, some studies suggest that much longer duration of pain, such as that verified 16 weeks after SNI in rats, is required for comorbid anxiety to occur (Seminowicz et al., 2009), if even ever occurring (Hubbard et al., 2015). It is important to consider that whether or not pain will influence anxiety-like behaviours may also depend on environmental- or study-related factors, like surgical technique, behavioural assays, and the choice of rodent sub-strain may also be involved.

Furthermore, although previous reports demonstrated that stress and PTSD can increase the probability of chronic pain development (Asmundson et al., 2000; Beck and Clapp, 2011; Dunne-Proctor et al., 2016; Sharp and Harvey, 2001; Sharp, 2004) or increase sensitivity to acute pain stimuli (Greenwood et al., 2016; He et al., 2013; Jennings et al., 2014; Nyland et al., 2015), the present results showed that CCI mice responded at lower thresholds to von Frey filaments regardless of their exposure to the snake. However, as the CCI mice responded to the lowest weight von Frey test filaments (i.e. 0.008g force), a floor effect may have obscured the effects of chronic stress.

In contrast, the snake-associated stressors produced striking effects on withdrawal reflex to mechanical stimuli applied by von Frey's test performed in the sham groups. Consistent with previous studies, the snake (Coimbra et al., 2006, 2017a), and later the snake plus its exuvia, induced antinociception responses in sham animals. In aversive situations, unconditioned fear-induced antinociception (Coimbra et al., 2006, 2017a; Cornélio et al., 2011; de Freitas et al., 2013, 2014*;* Heinricher et al. 2009; Mendes-Gomes and Nunes-de-Souza, 2005, 2009; Mendes-Gomes et al., 2011a,b) has an important adaptive effect, as it permits the exhibition of defensive reactions, such as freezing and flight/escape behaviour, even when an injury has occurred, increasing the animal's chances of survival (Bolles and Fanselow, 1980;

Butler and Finn 2009). Thus, after threat exposure, the sham (no CCI) group showed a strong antinociceptive response. However, approximately one week after the initial direct exposure to the snake and its exuvia and both before and after reexposure to the aversive experimental context, threatened sham groups responded to the lowest level of mechanical stimuli, the thinnest von Frey filaments. This observation represents an unusual set of differences from control levels unfolding over time; an initial increase in antinociception after exposure to threat stimuli followed later by a decrease in this initial antinociception, with sham animals showing allodynia that persisted after an additional reexposure to threat. However, the exposure of rodents to a single prolonged stressful (SPS) stimulus (Jennings et al., 2014; Zang et al., 2012), another experimental model of PTSD, has already been demonstrated to induce thermal hyperalgesia and mechanical allodynia, as measured by paw withdrawal latencies to a heat stimulus and the von Frey test, respectively. These alterations in nociceptive responses, displayed by rats never previously submitted to a nociceptive test, were verified as early as 7 days after the initiation of a SPS and lasted the length of the study, 28 days (Zang et al., 2012). Moreover, although it may be unknown whether patients with PTSD develop hypersensitivity to non-painful stimuli, it is known that hyperarousal is one of the key findings in PTSD, and it may represent a heightened response to an incoming somatosensory stimulus (Moeller-Bertram et al., 2014).

Unlike the fear-induced antinociception observed in Sham mice, CCI mice did not exhibit antinociception when exposed the snake, but continued to exhibit allodynia. Such data corroborate the hypothesis that in some states of chronic pain, inhibition of descending inhibitory systems and/or activation of the facilitatory pain system may occur (Heinricher et al., 2009, Jennings et al., 2014).

A number of animal models for PTSD have been proposed (Bertaina-Anglade et al., 2017; Campos et al., 2013; Goswami et al., 2013; Matar et al., 2006; Perrine et al., 2016; Sillivan et al., 2017, Schoner et al., 2017; Zang et al., 2012). A common feature of these models, reflecting attempts to parallel the chronic nature of PTSD, is that the post-traumatic behavioural response tends to be relatively durable, persisting past the initial or effective exposure event. However, the persistence seen in these models typically involves the consistent maintenance of an enhanced responsivity to stressful events, not an inversion of it, over time. In this context, increased responsivity to von Frey filaments, for threatened compared to non-threat-exposed mice, may be

seen as a new and different but also deviant response 6 days following the last exposure to the predator and its exuvia. Such delayed symptom onset is common in PTSD and appears to be relatively specific to that diagnosis (e.g. Tomb, 1994).

There are other findings in this study that suggest a link to PTSD. PTSD symptoms often include physical reactivity after exposure to traumatic reminders and heightened startle reactions (Brunello et al., 2001; Nemeroff et al., 2006, van der Kolk, 2001), both of which characterise the predator-exposed animals in this study following the initial, although not the subsequent, reexposure. In addition, the likelihood of PTSD appears to vary with the number/types of traumatic experiences to which the individual is exposed (e.g. Bender et al., 2015; Boasso et al., 2015), with (different types of) symptoms emerging only after additional exposures to the snake and its exuvia. Additionally, in PTSD, the emotional response pattern appears to show some degree of generalisation to different stimuli strongly associated with the traumatic experience or even with mild stressor stimuli not primarily associated with the trauma, and exacerbated responsiveness displayed by PTSD patients persists to the traumatic event itself (Monti and Smith, 1976; Morey et al., 2015; Osborne et al., 1975). Accordingly, snake exposure reduced time spent in the open-arm of the EPM test, an environment not associated to the previous aversive place, the enriched polygonal arena for snakes, where psychologically traumatic emotions were experienced by prey in the presence of the wild snake. Notably, the present findings of reduced duration of time spent in open-arms of the EPM test and increased risk assessment to the threat-associated situation as well as to the threat itself both suggest enhanced anxiety, as is frequently associated with PTSD (Brunello et al., 2001; Koenen et al., 2003; Lancaster et al., 2016; Puetz et al., 2015; Sipos et al., 2014).

In humans, the great majority of PTSD symptoms are currently accepted as mainly subjective (American Psychiatric Association, 2013), and there are no currently accepted approaches for the measurement of subjective events in animal models to study PTSD. Nonetheless, it is important to consider the findings outlined above, in addition to the primary requirement that post-psychological trauma-induced behavioural changes reflect an aversive/ traumatic event effect on the limbic system (Paschoalin-Maurin et al., 2018). Thereby, the present findings may reinforce the traumatic psychological clues, inherent to the present exposure paradigm in which the mice are exposed to the snake and exuvia, with correspondences between repeated exposure to different threatening situations and PTSD symptoms.

In conclusion, this set of psychological parallels between the specifically behavioural symptoms of PTSD displayed by humans and the aversive stimulus-related panic attack-like behavioural responses displayed by threatened prey suggests that this snake/exuvia/reexposure procedure may constitute a useful animal model to study PTSD. In this context, it will be of interest to determine the physiological and endocrine changes that this paradigm may involve over time, as well as the potential links between these behaviour changes and other disorders that are frequently comorbid with PTSD, such as depression and anxiety.

4. EXPERIMENTAL PROCEDURES

4.1. Animals

Male C57BL/6 mice (N = 23 and 35 for experiments 1 and 2, respectively) from the animal facility of the Ribeirão Preto Medical School of the University of São Paulo (FMRP-USP), weighing 28–30 g, were used. The mice were housed five per homecage (30 x 20 x 15 cm) with food and water available *ad libitum* in a temperature-controlled room (23 ± 1 ºC) under a 12-h/12-h light/dark cycle (lights on at 7 a.m.). The predators were wild constrictor rainbow Boidae snakes (*Epicrates cenchria crassus*; Reptilia; Boidae), weighing 800–2000 g (N = 2). The snakes were individuals of a species endemic of the Brazilian Southeast and were maintained in captivity in snake pits in the animal house of FMRP-USP (licensed by the Brazilian government; *Instituto Brasileiro do Meio Ambiente e de Recursos Naturais Renováveis* (IBAMA) Committee process 1/35/1998/000846-1). Two days before the experiments, the snakes were moved to a walled sun-lit field with appropriate shelter, grass, and water sources in the Laboratory of Neuroanatomy and Neuropsychobiology of the Ribeirão Preto Medical School of the University of São Paulo (LNN-FMRP-USP)/ Behavioural Neurosciences Institute (INeC) ophidiarium, licensed by the Brazilian government (IBAMA 3543.6986/2012-SP and 3543.6984/2012-SP processes) and by the São Paulo State government (*Secretaria do Meio Ambiente* (SMA)/ *Departamento de Fauna* (DeFau) 15.335/2012 process; Mechanisms of Defensive Behaviour and Unconditioned fear-induced antinociception in Snake-threatened Animals (MEDUSA) Project, *Sistema de Autorização e Informação em Biodiversidade* (SISBIO) authorisation for activities with scientific purposes 41435-1 process; SIGAM authorisation of installation process 39.043/2017; *Sistema Integrado de Gestão*

Ambiental (SIGAM) authorisation for use and handling of wild snakes process 39.044/2017). The snake enclosure in the LNN-FMRP-USP is illuminated by natural sunlight and fluorescent ultraviolet irradiation (ReptiSun; 20 W; 5UVB; Zoo Med Laboratories, San Luis Obispo, CA, USA) on rainy days and has artificial waterfalls and lagoons, natural rocks, and both tropical and artificial plants. The enclosure was kept under a 12-h/12-h light/dark cycle (lights on from 7:00 AM to 7:00 PM) at a constant room temperature of 25±1 ºC and 40–70% humidity. The snakes were fed at two specific times: once every 24 h with mice previously killed in CO_2 and once immediately before the start of each experiment with a live mouse of the same species and strain used in the study, aiming to decrease the risk of actual attacks. The feeding of the snake reduced the likelihood of attack, but still intimidating for the mice. The experiments were performed in accordance with the recommendations of the Commission of Ethics in Animal Experimentation of FMRP-USP (process 190/2015), which abides by the ethical principles in animal research adopted by the National Council for Animal Experimentation Control (CONCEA) and was approved by FMRP-USP Committee for Ethics in Animal Experimentation (CEUA) on 5/2/2016.

4.2. Experimental Protocol: Experiment 1

Experiment 1 was aimed at determining whether the snake exuvia, with its natural odour, was able to induce unconditioned fear-like behaviours. Naïve mice were habituated for two days in a polygonal (rectangular parallelepiped-shaped) transparent three-dimensional acrylic arena (140 cm in length, 62 cm in width and 50 cm in height) composed of seven faces, all of which are parallelograms (Coimbra et al., 2017a,b), with free access to food and water (Figure 7A). The floor of the arena was made of a transparent acrylic sheet placed on a stainless-steel platform. The floor was divided by red lines into 20 equal rectangles (4.2-mm width; Pritt mark-it). To minimise vibratory stimuli, the entire apparatus was placed on a granite surface (150 x 85 x 2 cm) that was elevated 83 cm above the floor of the laboratory. A burrow (shelter box: 10 x 7 x 5 cm) with black acrylic walls was placed in one corner of the arena. The burrow had one entrance with a 2-cm diameter, allowing the rodents to enter and exit the burrow. The lid of the burrow was made of translucent acrylic to facilitate the recording of mouse behaviour inside the burrow. Three translucent acrylic stairs, with a small platform (7 x 4 x 10.5 cm) at the top, were provided in the arena, one in the

corner beside the burrow, another in the opposite side, and the third one, in the arena sidewall (Almada and Coimbra, 2015; Almada et al., 2015).

On the third day, the bedding, food and water were removed from the polygonal arena, and mice were individually exposed for 10 min to the same arena to which they had been habituated. However, at this time, the polygonal arena contained a snake (N = 7 mice/group) or only its exuvia (N = 9 mice/group), i.e., layer of skin shed during ecdysis, which contains the snake natural odour, without any barrier preventing direct contact with the predator. A control group (N = 7 mice/group) was similarly habituated and exposed to the polygonal arena, but not confronted with the snake or its moulted skin. The exuvia was always put back in the snake cage and kept with the snake for 48 h before the next experiment. It is also important to note that, although different sample sizes have been used in each experimental group, there were no drop outs or accidental deaths during the study, and no mouse was harmed by the snakes during the current investigation.

During exposure to the arena (with or without the predator or its exuvia), the frequency and/or duration of the following behaviours were recorded: (a) risk assessment, including (a1) the stretch attend posture, in which the body is stretched forward but the animal's hind paws remain in position, followed by retraction to the original position, (a2) flat back approach, where the mouse slowly moves forward with the body stretched, and (a3) defensive attention or alertness, which is an interruption of the ongoing behaviour for less than 6 s to occasionally scan the environment or sniff the air; (b) defensive immobility or freezing, defined as the absence of movements, except those related to breathing, for at least 6 s, with the animals potentially presenting neurovegetative reactions, such as exophthalmia, defecation and/or micturition; (c) time in protected areas, which were below or on top of the stairs and inside or on top of the burrow; and (d) escape, which included running or jumping towards the stairs and/or burrow or other places of the arena without protected areas (Almada et al., 2015; Blanchard et al., 1993; Coimbra et al., 2017a; Dalvi and Rodgers, 1996; Kalueff and Tuohimaa, 2005; Nunes-de-Souza et al., 2002, Sorregotti et al., 2013).

4.3. Experimental Protocol: Experiment 2

4.3.1. Model of neuropathic pain

The animals were submitted to a procedure in which the sciatic nerve was lesioned by its chronic constriction (CCI) (N = 18), as described by Bennett and Xie (1988) and modified by Sommer et al. (1995). However, that procedure causes Wallerian degeneration in the lesioned nerve with several sensorial impairments and autotomy. For this reason, instead of three or four ligations, the animals received only one constriction of the peripheral nerve (Dias et al., 2013; Medeiros et al., 2020). The tension generated in this ligation was mild, only enough to cause a mild ischaemia, without interrupting blood flow completely.

Before surgery, the animals were anaesthetised with an intraperitoneal injection of 10% ketamine (100 mg/kg) in 2% xylazine (10 mg/kg). In anaesthetised mice, a longitudinal incision in the proximal third of the thigh at the dorsolateral region and trochanter/femur level was made. The longitudinal muscle layer was then gently divided by blunt dissection with scissors and other microsurgery instruments. A single ligature with chrome catgut 4-0 thread was performed around the right sciatic nerve proximal to its trifurcation until the diameter of the nerve was slightly constricted (Dias et al., 2013). The incision in the skin was sutured with braided silk surgical thread 4-0. The animals were then treated with an intramuscular injection of penicillin G-benzatine (120.000UI/0.1 mL) and maintained in post-operative recovery in their home cages. The sham group (N = 17) underwent the same surgical procedures without CCI. Sham and CCI mice were never housed together in the same home cages, since studies demonstrate that hyperalgesia can be observed in “bystander” mice housed and tested in the same room as mice subjected with inflammatory or neuropathic pain (Baptista-de-Souza et al., 2015; Langford et al., 2006; Smith et al., 2016).

4.3.2. Test of mechanical allodynia

To evaluate the nociceptive threshold, von Frey filaments were used (Cunha et al., 2004; Möller et al., 1998; Prado et al., 2002; Vivancos et al., 2004) in all experimental groups The mice were individually placed in acrylic cages on a wire grid floor and a series of von Frey filaments were used to determine the threshold of response to the mechanical stimulus. Each filament was applied with a mild force for approximately 3-4 s. If the animal did not shake, lick or withdraw the paw, another filament with greater diameter and force was used until a response was observed. Once the animal responded to a determined filament, two other confirmatory recordings were made with the same filament, with an interval of approximately 10 s

between each measure. All mice responded when stimulated with a maximum of 9 different filament forces that ranged from 0.008 to 1.4 in grams force.

The von Frey test was performed before the Sham surgery or surgery for constriction of the sciatic nerve (CCI), and 10 days after surgery. Also, von Frey test was applied in the 22nd day after surgery, at the following times: 1 h before and 0, 15, 30, 45 and 60 min after exposure to the polygonal arena (with or without the presence of the predator and its exuvia). In addition, this sequence was repeated before and after the reexposure to the polygonal arena (with or without the predator's exuvia).

4.3.3. Exposure of mice to an aversive environment

Nineteen days after CCI, the mice were habituated in groups of approximately 12 animals, from three different homecages, for two days in the same polygonal arena enriched with two elevated platforms for escape and a burrow, used in Experiment 1 (see 4.2 and Figure 7A). However, it is important to highlight that we never habituated together Sham and CCI mice, for the same reasons mentioned in the last sentence of the item 4.3.1.

Twenty-one days after being submitted to surgery for sciatic nerve lesion, Sham (n = 8) or CCI (n = 9) mice were individually placed for 4 h in fenestrated transparent acrylic boxes positioned in the interior of the polygonal arena, and a constrictor snake (*Epicrates cenchria crassus*; Reptila; Boidae) was placed on the upper surface of these boxes (Figure 7B). The snake could move freely on the fenestrated ceiling of boxes containing each mouse in isolation inside entirely fenestrated chambers. These chambers were placed side-by-side covering all the surface of the floor of the polygonal arena. After this procedure, the animals were put back in their homecages. After 24 h, responses to mechanical stimuli were evaluated (von Frey test) in mice for a baseline withdrawal response recording, and 1 h later, mice were individually exposed for 10 min to the enriched polygonal arena with escape elevated platforms and a burrow, in the presence of the snake and its exuvia, without any barrier to prevent direct contact between prey and the predator (Figure 7C and 7D). Two control groups, one with Sham (n = 9) and one with CCI (n = 9) animals, were similarly exposed to the polygonal arena but not confronted by the snake and its moulted skin.

During arena exposure (with or without the predator and its exuvia), the frequency and duration of the following behaviours were recorded: risk assessment, defensive immobility or freezing, time in protected areas and escape (for definition of

these behaviours, see 4.2). Moreover, the following other behaviours were also recorded: grooming; rearing, defined as vertical movement against the walls; and crossings, defined as the frequency of crossings over each rectangle drawn on the floor of the arena (Almada et al., 2015; Blanchard et al., 1993; Dalvi and Rodgers, 1996; Kalueff and Tuohimaa, 2005; Nunes-de-Souza et al., 2002, Sorregotti et al., 2013).

Immediately after exposure to the polygonal arena, the mice were submitted to the von Frey test 5 times, with an intertest interval of 15 min. After 6 days, mice that were confronted by the snake and its exuvia were individually reexposed for 10 min to the polygonal arena containing only the exuvia (Figure 7E and 7F). Regarding animals that were not previously confronted by the predator and its exuvia, neither the predator nor its exuvia were present during the reexposure procedure. During reexposure to the arena, the frequency and/or duration of the same behaviours recorded during the initial exposure were recorded. Immediately after reexposure, the mice were again submitted to the von Frey test 5 times with the same 15-min intertrial intervals.

4.3.4. Elevated plus-maze (EPM) test

Considering that patients with PTSD tend to be more anxious (Lee et al., 2016), the present study determined whether the same phenomenon could be observed in mice after snake confrontation. Twenty-four hours after the reexposure to the aversive context, the rodents were submitted to the EPM test for 5 min. This animal model of anxiety was originally described by Handley and Mithani (1984) and Pellow et al. (1985) using rats as experimental subjects and was subsequently validated for mice (Lister, 1987; Stephens et al., 1986). This test is based on the natural fear displayed by rodents of open places and is frequently used to evaluate anxiety-related behaviours as well as the anxiolytic or anxiogenic properties of drugs. The apparatus is grey and made with acrylic. The EPM consists of two open (30.7 x 6 x 0.5 cm) and two closed arms (30.7 x 6 x 15.5 cm) connected to a common central platform (6 x 6 cm) and raised to a height of 38.5 cm above floor level. Anxiety was assessed by analysing the percentages of open arm entries [(open/total) × 100] and time spent in the open arms [(open arm time/300) × 100]. The frequency of closed arm entries was used to measure the locomotor activity. It is important to highlight that the same

experimenter (that performed all the Experiment 1 and 2) exposed the animals to the EPM. However, after placing the mouse in the EPM, the researcher left the room, and the mouse behaviour was video-recorded for a later analysis.

To summarise the experimental protocol, a timeline of all the experimental procedures that the mice experienced in Experiment 2 is presented in Table 1.

4.4. Statistical Analysis

In experiment 1, the data were analysed by one-way analysis of variance (ANOVA). In experiment 2, the behavioural data were analysed by either a repeated measure two-way analysis of variance (MANOVA) or three-way MANOVA to evaluate the effects of the nerve injury (*Sham* vs. CCI), presence of the snake and its exuvia, the experimental context (exposure vs. reexposure) and the interaction among these factors. For the statistical analysis of mechanical allodynia, a three-way repeated measures MANOVA was used. In all cases, significant effects of ANOVA and MANOVA were followed by Bonferroni's *post hoc* test. Values of $P \leq 0.05$ were considered statistically significant.

Table 1: Timeline of the experimental procedures. **Yes** indicates that this group of mice was submitted to the conditions described in the header, whereas **No** indicates the opposite. VF1 and VF2-VF6 indicate the first and second to sixth von Frey test measures.

Day / **Procedure** / **Groups (n)**	**1st VF1/ Sham or CCI surgery**	**10th VF 1**	**19th and 20th habituation to the arena**	**21st allocation to the acrylic boxes with a snake on the upper surface for 4h**	**22nd VF1/ exposure to the arena with the snake and its exuvia for 10 min/ VF2-VF6**	**28th VF1/ reexposure to the arena with the exuvia for 10 min/ VF2-VF6**	**29th EPM for 5 min**
Sham Non-threated (9)	VF and Sham surgery	Yes	Yes	Yes, but without snake	Yes, but without snake	Yes, but without exuvia	Yes

					and exuvia		
Sham Threated (8)	VF and Sham surgery	Yes	Yes	Yes	Yes	Yes	Yes
CCI Non-threatened (9)	VF and CCI surgery	Yes	Yes	Yes, but without snake	Yes, but without snake and exuvia	Yes, but without exuvia	Yes
CCI Threatened (9)	VF and CCI surgery	Yes	Yes	Yes	Yes	Yes	Yes

AUTHORS CONTRIBUTIONS

J. Mendes-Gomes performed the experiments, analysed data and wrote the manuscript; Paschoalin-Maurin handled and fed the snakes; L.F. Donaldson, B.M. Lumb, and D.C. Blanchard interpreted data and wrote the manuscript; N.C.Coimbra designed the experiments, designed the enriched polygonal arena for snakes versus prey confrontations, and the current post-traumatic stress disorder apparatus, interpreted data, and wrote the manuscript.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest with respect to the work presented herein.

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Figure Captions

Figure 1: Frequency and duration of defensive-like behaviours in a 10-minute period exhibited by naïve C57BL/6 mice (n = 7-9/group) exposed to a polygonal arena without (non-threatened) or with a snake or its exuvia. Data are presented as the mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. $^{a}P < 0.05$, mice exposed to the snake or to its exuvia vs. non-threatened mice; $^{b}P < 0.05$, mice exposed to the snake vs. those exposed to the exuvia.

Figure 2: Frequency and duration of defensive-like behaviours in a 10-minute period exhibited by C57BL/6 mice (n = 8-9/group), with (CCI) or without (sham) sciatic nerve constriction, exposed to a polygonal arena without (non-threatened) or with a snake and its exuvia (threatened) and, 6 days later, reexposed to the aversive experimental context, i.e., the arena without (non-threatened) or with the exuvia (threatened). Data are presented as the mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. $^{a}P < 0.05$, sham threatened group vs. sham non-threatened group during exposure; $^{b}P < 0.05$, CCI threatened group vs. CCI non-threatened group during exposure; $^{c}P < 0.05$, sham threatened group vs. sham non-threatened group during reexposure. $^{d}P < 0.05$, CCI threatened group vs. CCI non-threatened group during reexposure. $^{e}P < 0.001$, within-groups comparison, exposure vs. reexposure.

Figure 3: Frequency and duration of non-defensive-like behaviours in a 10-minute period exhibited by C57BL/6 mice (n = 8-9/group), with (CCI) or without (sham) sciatic nerve constriction, exposed to a rectangular arena without (non-threatened) or with a snake and its exuvia (threatened) and, 6 days later, reexposed to the aversive

experimental context, i.e., arena without (non-threatened) or with the exuvia (threatened). Data are presented as the mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. [a]$P < 0.05$, sham threatened group vs. sham non-threatened group during exposure; [b]$P < 0.05$, CCI threatened group vs. CCI non-threatened group during exposure; [c]$P < 0.05$, sham threatened group vs. sham non-threatened group during reexposure; [d]$P < 0.05$, CCI threatened group vs. CCI non-threatened group during reexposure. [e]$P \leq 0.05$, within-groups comparison, exposure vs. reexposure.

Figure 4: Withdrawal thresholds to the von Frey filaments in C57BL/6 mice (n = 8-9/group) before and 10 days after being submitted (CCI) or not (sham) to sciatic nerve constriction. Importantly, an increase in responsivity is shown by a decrease in the pressure needed to elicit a withdrawal response. Data are presented as the mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. [a]$P < 0.0001$, compared to the baseline measure. [b]$P < 0.05$, compared to the sham group.

Figure 5: Withdrawal thresholds to the von Frey filaments, measured at a number of time intervals, in C57BL/6 mice (n = 8-9/group) submitted or not to sciatic nerve constriction (CCI), exposed to the polygonal arena for snakes (without or with a snake and its exuvia) (A), and 6 days later, reexposed to the experimental context i.e., arena without (non-threatened) or with the exuvia (threatened) (B). Importantly, an increase in responsivity is shown by a decrease in the pressure needed to elicit a withdrawal response. Data are presented as the mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. [a]$P \leq 0.001$ compared to the baseline measure, obtained before the confrontation to the predator; [b]$P \leq 0.01$, sham threatened group vs. sham non-threatened group; [c]$P < 0.05$, CCI threatened group vs. sham threatened group; [d]$P < 0.0001$, CCI non-threatened group vs. sham non-threatened group.

Figure 6: Percentages of open-arm entries (A) and time (B) and frequency of closed-arms entries (C) of sham and sciatic nerve constriction (CCI) mice (n = 8-9/ group) exposed to the elevated plus maze (EPM) six days after exposure to a polygonal arena for snakes (without or with a snake and its exuvia) and one day after reexposure to the experimental context (arena without or with the exuvia). Data are presented as the

mean ± S.E.M., and comparisons between groups were performed using Bonferroni's post hoc test. [a]$P < 0.05$ compared to the sham non-threatened group. [b]$P < 0.05$ compared to the CCI non-threatened group.

Figure 7: Photographic documentation of habituation procedure (A); exposure of C57BL/6 mice, in isolation inside fenestrated and transparent compartments, to the *Epicrates cenchria crassus* constrictor snakes (B); and representative aversive stimulus-induced unconditioned (C and D) and conditioned (E and F) fear-related behavioural responses displayed by *Mus musculus* confronted with *Epicrates chenchria crassus* in the enriched polygonal arena for snakes. Defensive immobility (freezing) under an elevated escape platform (C) and inhibitory avoidance and stretch attend posture after oriented escape to the burrow (D) were displayed by prey during confrontation with predator. Flat back approach/interactions between prey and the exuvia (moulted skin with the smell of the snake) (E) and defensive immobility displayed by prey on the elevated platform (F) were showed by prey during exposure to the experimental context with the exuvia, but without the predator.