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Original Article

Effect of Sciatic Nerve Transection on acetylcholinesterase activity in spinal cord and skeletal muscles of the bullfrog *Lithobates catesbeianus*

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(With 3 figures)

Abstract

Sciatic nerve transection (SNT), a model for studying neuropathic pain, mimics the clinical symptoms of "phantom limb", a pain condition that arises in humans after amputation or transverse spinal lesions. In some vertebrate tissues, this condition decreases acetylcholinesterase (AChE) activity, the enzyme responsible for fast hydrolysis of released acetylcholine in cholinergic synapses. In spinal cord of frog *Rana pipiens*, this enzyme's activity was not significantly changed in the first days following ventral root transection, another model for studying neuropathic pain. An answerable question is whether SNT decreases AChE activity in spinal cord of frog *Lithobates catesbeianus*, a species that has been used as a model for studying SNT-induced neuropathic pain. Since each animal model has been created with a specific methodology, and the findings tend to vary widely with slight changes in the method used to induce pain, our study assessed AChE activity 3 and 10 days after complete SNT in lumbosacral spinal cord of adult male bullfrog *Lithobates catesbeianus.* Because there are time scale differences of motor endplate maturation in rat skeletal muscles, our study also measured the AChE activity in bullfrog tibial posticus (a postural muscle) and gastrocnemius (a typical skeletal muscle that is frequently used to study the motor system) muscles. AChE activity did not show significant changes 3 and 10 days following SNT in spinal cord. Also, no significant change occurred in AChE activity in tibial posticus and gastrocnemius muscles at day 3. However, a significant decrease was found at day 10, with reductions of 18% and 20% in tibial posticus and gastrocnemius, respectively. At present we cannot explain this change in AChE activity. While temporally different, the direction of the change was similar to that described for rats. This similarity indicates that bullfrog is a valid model for investigating AChE activity following SNT.

Keywords: acetylcholinesterase, sciatic nerve transection, tibial posticus, gastrocnemius, spinal cord, frog.

Efeito da Secção do Nervo Isquiático sobre a atividade da acetilcolinesterase em medula espinal e músculos esqueléticos da rã-touro *Lithobates catesbeianus*

Resumo

A transecção do nervo isquiático (SNT), um modelo para estudar dor neuropática, simula os sintomas clínicos do "membro fantasma", uma condição dolorosa que ocorre nos humanos após amputação ou secção completa da medula espinal. Essa condição muda a atividade da acetilcolinesterase (AChE), a enzima responsável pela rápida hidrólise da acetilcolina liberada nas sinapses colinérgicas, em alguns tecidos de vertebrados. Em medula espinal de rã *Rana pipiens*, a atividade da AChE não foi significativamente alterada nos primeiros dias após a secção da raiz ventral, outro modelo para o estudo da dor neuropática. Uma questão ainda não respondida é se a SNT diminui a atividade da AChE na medula espinal de rã *Lithobates catesbeianus*, uma espécie que vem sendo usada como modelo em estudos da dor neuropática induzida por SNT. Como cada modelo animal é criado a partir de metodologia específica, e seus resultados tendem a variar com pequenas mudanças na metodologia de indução da dor, o presente estudo avaliou a atividade da AChE em medula espinal lombossacral de rã-touro *Lithobates catesbeianus*, adultos, machos, 3 e 10 dias após a completa SNT. Como há diferenças temporais na maturação de placas motoras em músculos esqueléticos de ratos, nosso estudo ainda demonstrou, na rã-touro, os efeitos da SNT sobre a atividade da AChE nos músculos esqueléticos tibial posticus, um músculo postural, e gastrocnêmio, um músculo frequentemente usado em estudos do sistema motor.

A atividade da AChE não mudou significativamente na medula espinal aos 3 e 10 dias após a SNT. Nos músculos, a atividade não alterou significativamente aos 3 dias após a lesão, mas reduziu de forma significativa aos 10 dias após a SNT. Aos 10 dias, a diminuição foi 18% no músculo tibial posticus e 20% no gastrocnêmio. No momento, nós não temos explicação para essa mudança na atividade da AChE. Embora temporalmente diferente, o sentido da mudança é similar ao que é descrito em ratos. Esta similaridade torna a rã-touro um modelo válido para se estudar questões ainda não respondidas da SNT sobre a AChE.

Palavras-chave: acetilcolinesterase, secção do nervo isquiático, tibial posticus, gastrocnêmio, medula espinal, rã.

1. Introduction

Contractile muscle activity is controlled by the motor neuron-muscle system. In this system, acetylcholine (Ach) released from the presynaptic nerve ending binds to its receptors in the postsynaptic membrane and causes its depolarization. This triggers an action potential and the muscle fibers contract. The action of acetylcholine must then be terminated to allow the transmission of the next nerve impulse. For this purpose, acetylcholinesterase (AChE), a membrane-bound enzyme localized in neuromuscular junctions of the skeletal muscles and cholinergic neurons in nervous tissue, is the responsible for fast hydrolysis of released acetylcholine in the synapses (Pawlowski et al., 2013; Trinkaus et al., 2008).

Studies have demonstrated that anticholinesterase agents, which increase Ach levels in the synaptic cleft, ameliorate neuropathic pain (Chen and Pan, 2003), which arises as a direct consequence of a lesion or disease affecting the somatosensory system (Treede et al., 2008). According to Bartolini et al. (2011), strategies to induce analgesia involving cholinergic mechanisms have been used for a long time, but in the modern times cholinergic-induced analgesia lost its importance due to numerous side effects. A reemergence of this therapeutic approach may happen from our improved understanding of the mechanisms through which this form of analgesia can be induced without aversive effects.

As neuropathic pain has multiple etiologies, different animal models have been created to study its pathophysiological mechanisms (Jaggi et al., 2011). Sciatic nerve transection (SNT) is the oldest model to study these mechanisms. This experimental situation is a model that mimics the clinical symptoms of "phantom limb", a pain condition that arises in humans after amputation or transverse spinal lesions (Jaggi et al., 2011; Klusáková and Dubový, 2009). Since the precise mechanisms for this pain are still unclear (Hsu and Cohen, 2013; Nikolajsen, 2012), animals models may contribute to our understanding of the pathogenesis of this pain.

The bullfrog *Lithobates catesbeianus* is an semiaquatic amphibian (Hedrick et al., 2015) native to the United States that was imported into Brazil in the early 1930s and has been raised in this country ever since (Rocha and Branco, 1998). This species has been used as a model for the study of SNT-induced neuropathic pain (Rigon et al., 2014, 2013; Guedes et al., 2004a, b; Partata et al., 2002) because it provides a phylogenetic perspective on the mechanisms of pain research (Rigon et al., 2013). These studies reported alterations that shared similarities with those observed in mammals, while others were unique to this animal species.

In mammals and birds, SNT cause temporary decrease in AChE activity in skeletal muscles and nervous tissue (Ijkema-Paassen et al., 2002; Wan et al., 1997). In frog *Rana pipiens*, it has been demonstrated that spinal-cord AChE activity did not significantly change in the first days following ventral root transection (Sinicropi et al., 1982), another model to study neuropathic pain (Jaggi et al., 2011; Klusáková and Dubový, 2009). An answerable question is whether SNT decreases AChE activity in spinal cord of *Lithobates catesbeianus*. According to Jaggi et al. (2011), each animal model has been created with a specific methodology, and the results tend to vary widely with slight changes in the methods used to induce pain; therefore, it is essential that data from different pain models be reported and interpreted in the context of the specific pain model. Thus, our study assessed the effect of SNT on AChE activity in lumbosacral spinal cord of *Lithobates catesbeianus*. We also assessed the effects of SNT on AChE activity in bullfrog tibial posticus and gastrocnemius muscles because SNT has been shown to induce time scale differences of motor endplate maturation in skeletal muscles of rats (Ijkema-Paassen et al., 2002). Tibial posticus was chosen because it is a postural muscle in frogs (Crockett and Peters, 2008), and gastrocnemius is a typical skeletal muscle that is frequently used to study the motor system. All experiments were performed 3 and 10 days after SNT because the changes in some molecules that have a modulatory role in pain are fully developed 3 days following peripheral nerve injury and some recovery appears to occur at day 10 (Guedes et al., 2004a, b; Partata et al., 2002; Sinicropi et al., 1982). We believe our results will determine if the changes in AChE activity are similar across amphibians and mammals, potentially increasing our knowledge of the effects of SNT on bullfrog tissues.

2. Material and Methods

2.1. Experimental animals

Experiments were conducted in 20 adult male *Lithobates catesbeianus* frogs, weighing 100-250 g. All animals procedures were approved by the Ethics Committee at the Federal University of Rio Grande do Sul, Brazil (# 2007768). The specimens were obtained from a local frog farm (RANASUL, Imbé/RS) in summer season (January and February months, where mean of temperature was 26.5 °C). At the farm, frogs were held at ambient temperature with free access to running tap water and were fed with specific food (Nutripeixe TC40,

Purina). Upon arrival at the laboratory frogs were housed in cages with water and kept under ambient temperature similar to the temperature found in farms. The animals were fed *ad libitum* with the same food offered in farms, which was now offered on a vibration plate to move the pellets. The animals remained in the laboratory for at least 2 weeks before being used in the surgery, as described by Rigon et al. (2013). The aquarium water was periodically replaced with fresh tap water.

2.2. Surgery

The SNT was made under anesthesia (prilocaine 0.1 mL/100 g body weight) and sterile conditions as described by Rigon et al. (2014, 2013). For nerve transection the right sciatic nerve was randomly exposed and completely transected approximately 3 mm distal to the sciatic notch. The transection injury was produced by first placing one silk ligature around the sciatic nerve in the upper thigh. The nerve was then cut adjacent to the silk ligature and the ends stayed free to facilitate the regeneration. This was done because the progression of events during both demyelination and recovery in cut axons are also under local control and can be carried out in the absence of constant communication with the nerve cell body in frog *Xenopus laevis* (Rubinstein and Shrager, 1990). In animals with the sham injury, the sciatic nerve was exposed as in the other injury, and gently manipulated. In all animals, the muscle and skin layer was immediately sutured with thread and a topical antibiotic (Rifocina Spray®, Sanofi-Aventis Farmacêutica Ltda, São Paulo, Brazil) applied. At 3 and 10 days after surgery, the animals were sacrificed (n=5/group).

2.3. Preparation of tissue samples

After the third and tenth day frogs were killed by decapitation and their lumbosacral spinal cord and tibial posticus and gastrocnemius muscles were quickly dissected out. The tissues were immediately frozen on dry ice and stored at -70 °C for further determination of AChE activity.

For assay, the samples were homogenized in 10 volumes 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1000 x g. The supernatant was used for the enzymatic AChE analysis.

2.4. Determination of AChE activity

Acetylcholinesterase activity was determined according to Ellman et al. (1961), with some modifications (Villescas et al., 1981). Hydrolysis rates were measured at acetylthiocholine (ACSCh) concentration of 0.8 mM in 1 mL assay solutions with 30 mM phosphate buffer, pH 7.5, and 1 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. Fifty microliters of supernatant were added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by the formation of the thiolate dianion of DTNB at 412 nm for 2-3 min (intervals of 30 s). Specific enzyme activity was expressed as µmol hydrolysis of acetylthiocholine (ACSCh) per hour per milligram of protein. All samples were run in duplicate.

2.5. Protein measurements

Protein quantification was performed using the Lowry method. Absorbance was measured at 650 nm with a spectrophotometer (Biospectro). Data, expressed as mg/mL, were obtained based on a standard curve using bovine serum albumin (Lowry et al., 1951).

2.6. Statistical analysis

The results were analyzed using two-way ANOVA (factors: lesion and time) followed by Bonferroni post-hoc test. Differences were considered statistically significant when P was <0.05. All statistical analysis was carried out with Sigma Stat 3.5 software.

3. Results

In the present study, SNT did not induce changes in bullfrog body weight. No significant change in AChE activity was found in tissues of the sham-operated group throughout the experimental period (Figures 1, 2, 3).

After SNT, the two-way ANOVA did not show significant differences (P = 0.085) in AChE activity in lumbosacral spinal cord at days 3 and 10 (Figure 1). In skeletal muscles, AChE activity did not show significant changes (P=0.063 for tibial posticus muscle; P=0.069 for gastrocnemius muscle) at day 3 (Figures 2, 3), but in tibial posticus and gastrocnemius muscles it was decreased by 16% and 10%, respectively, as compared to sham groups. At day 10, SNT induced a significant decrease in AChE activity in both these muscles. For tibial posticus muscle, the two-way ANOVA showed significant difference between groups (P<0.012) but not between time after surgery (P=0.272). For gastrocnemius muscle, the two-way ANOVA showed significant difference between groups (P=0.048) and time after surgery (P=0.024). The reduction was of about 18% and 20% in tibial posticus muscle and gastrocnemius muscles, respectively, as compared to sham groups (Figures 2, 3).

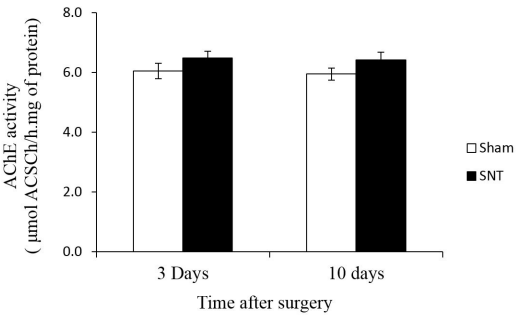

Figure 1. Acetylcholinesterase (AChE) activity in spinal cord from sham and transected frogs. The tissue was collected from sham-operated (Sham) and axotomized (sciatic nerve transection, SNT) frogs. Data are given as mean+S.E.M (n = 5 for each group). There was not any significant difference between the groups (P>0.05, two-way ANOVA).

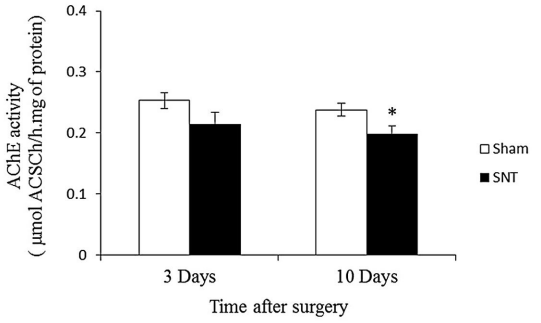

Figure 2. Acetylcholinesterase (AChE) activity in tibialis posticus muscle from sham and transected frogs. The tissue was collected from sham-operated (Sham) and axotomized (sciatic nerve transection, SNT) frogs. Data are given as mean+S.E.M (n = 5 for each group); *indicates significant difference compared to sham group at the same time point and groups at day 3 (P<0.05, two-way ANOVA followed by Bonferroni's post hoc test).

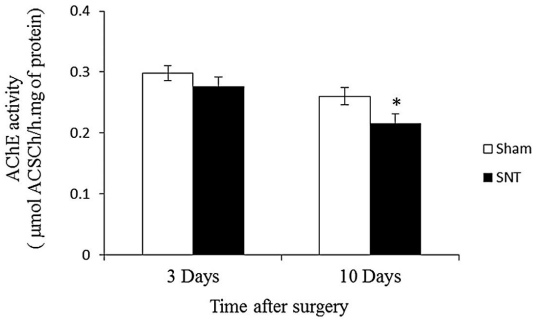

Figure 3. Acetylcholinesterase (AChE) activity in gastrocnemius muscle from sham and transected frogs. The tissue was collected from sham-operated (Sham) and axotomized (sciatic nerve transection, SNT) frogs. Data are given as mean+S.E.M (n = 5 for each group); *indicates significant difference compared to sham group of the period and sham group at 3 days (P<0.05, two-way ANOVA followed by Bonferroni's post hoc test).

It should be noted that AChE activity was about 25 times higher in lumbosacral spinal cord than in skeletal muscles. In skeletal muscles, the highest AChE activity was found in gastrocnemius muscle, where it was about 12% higher than in tibial posticus.

4. Discussion

The main finding in the present study was the lack of significant change in AChE activity in bullfrog lumbosacral spinal cord, while a significant decrease was found in this enzyme's activity in skeletal muscles 10 days after SNT. Since SNT is a model of neuropathic pain (Jaggi et al., 2011; Klusáková and Dubový, 2009), it would be interesting to have assessed the mechanical and thermal sensitivities in frogs with SNT, as these measures are indicators of nociceptive activity (Willenbring and Stevens, 1997). At the moment it was impossible for us to carry out these analyses.

In our study, a naive group (i.e. an intact group) was not used because SNT did not induce significant changes in sham-operated frogs (Rigon et al., 2014; Burgess and McIlwain, 1994; Sinicropi et al., 1982). In addition, our AChE activity values showed a similar range as previously described for spinal cord of frog *Rana pipiens* (Sinicropi et al., 1982).

Unchanged AChE activity in spinal cord after nerve injury was described as well for frog *Rana pipiens* (Sinicropi et al., 1982). In that study, AChE activity fell slightly below normal by day 35 and returned to normal by day 75 after ventral root transection. A similar period could be necessary to induce decrease in AChE activity in bullfrog spinal cord after SNT. Since no significant change was found in AChE activity of the mammalian spinal cord after peripheral nerve injury (Malatová et al., 1985), it may be suggested that this experimental condition induces a similar response in spinal cord AChE activity in both frogs and mammals. However, further studies are required to clarify this suggestion. It was demonstrated that there are differences between frog and rat concerning molecular forms of AChE. While the 18S form of AChE accounted for 9-30% of total AChE activity in frog central nervous system, the 16S or 20 S forms, homologous to 18S, accounted for 1-6% of total AChE activity in rat central nervous system (Nicolet and Rieger, 1982). Thus, it appears important to demonstrate the effect of SNT on distinct molecular forms of AChE in bullfrog spinal cord to better characterize this experimental model. In rat spinal cord, SNT induced a decrease in proline-rich membrane anchor (PRIMA) of the globular form of AChE (Leung et al., 2009). However, the effect of SNT on this molecular form of AChE in frog spinal cord is unknown.

However, a significant decrease in AChE activity occurred in bullfrog skeletal muscles at day 10. This is in line with a previous study where homogenates prepared from frog muscles of the posterior extremity were used (Pytasz et al., 1977). According to these authors, the skeletal muscle denervation causes an initial rise in AChE activity within 30 hours, followed by its fall to control values after 4-6 days. Afterwards, it gradually decreases and reaches the lowest values at day 15. Curiously, our study found a tendency to reduction in muscle AChE activity at day 3. This difference may be due to the separation of the two skeletal muscles in our study. However, other factors should be considered, such as seasonal temperature changes. In *Rana temporaria*, signs of nerve sprouting and regression are infrequent in winter and frequent in summer (Wernig et al., 1980). In our study, AChE activity was measured in summer, when the mean ambient temperature was 26.5 °C, whereas in Pytasz et al. (1977) study it was measured in frogs maintained at 15-18 °C. As the highest AChE activity was observed in summer, decreasing significantly below 45% in winter in freshwater fish

Cnesterodon decemmaculatus (Menéndez-Helman et al., 2015), we cannot exclude the possibility that the difference in AChE activity in bullfrog muscles between our findings and those reported by Pytasz et al. (1977) may be related to seasonal changes in muscle activity.

Interestingly, the effect of SNT on AChE activity in bullfrog skeletal muscles was similar to the one described for mammals (Glisović et al., 2010; Ijkema-Paassen et al., 2002; Guth et al., 1964). However, while SNT induced a rapid initial decrease in AChE activity in rat skeletal muscles (Guth et al., 1964), such reduction occurred later and more slowly in bullfrog tissues. We suggest that such difference may be related to the nervous system's slower metabolic rate in frogs than in mammals (McDougal Junior et al., 1968). Nevertheless, this difference does not preclude the use of frogs as an experimental model to study the effect of SNT on cholinergic synapses. According to Rigon et al. (2013), the temporal differences between frog and rat highlight the necessity to know the effects of SNT on frog nervous tissue in order to better explore this experimental model. Since the direction of AChE changes was similar in spinal cord and skeletal muscles from both bullfrog and rat, this finding reinforces the use of bullfrog as an experimental model to answer still unanswered questions about AChE after SNT. Since anticholinesterase agents ameliorate neuropathic pain (Chen and Pan, 2003), the similarity in AChE changes between bullfrog and rat also indicates bullfrog as a valid experimental model to investigate the effects of distinct anticholinesterase agents in neuropathic pain, which may contribute to a better clinical use of these drugs to treat this pain. However, the study has limitation. Since the events during both demyelination and recovery in cut axons are also under local control and can be carried out in the absence of constant communication with the nerve cell body in frog *Xenopus laevis* (Rubinstein and Shrager, 1990), we cannot exclude the contribution of reinnervation in our results. The decrease in AChE activity in skeletal muscles at day 10 may be indicating lack of regeneration, but more studies are necessary to confirm this suggestion. Thus, in future studies, this question should be considered.

5. Conclusion

Our study showed that SNT did not induce significant changes in AChE activity in lumbosacral spinal cord of the bullfrog at days 3 and 10. While no significant change was found in AChE activity of the tibial posticus and gastrocnemius muscles 3 days after SNT, a significant decrease occurred at day 10. Despite more temporally slower, these changes were similar to those described for mammals, which reinforce the use of bullfrog as an experimental model to study still unanswered questions about AChE after SNT. Since the temporal difference only highlights the necessity to know the effects of SNT on frog nervous tissue to better explore this experimental model, future studies should consider the temporal peculiarities demonstrated here.

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