Regional brain c-fos activation associated with penile erection and other symptoms induced by the spider toxin Tx2-6

Lanfranco R.P. Troncone a,*, Katherine G. Ravelli a, Fabio C. Magnoli c, Ivo Lebrun b, Debora C. Hipolide d, Roger Raymond e, José N. Nobrega e

a Laboratory of Pharmacology, Instituto Butantan, São Paulo, Brazil
b Laboratory of Biochemistry, Instituto Butantan, São Paulo, Brazil
c Laboratory of Immunochemistry, Instituto Butantan, São Paulo, Brazil
d Department of Psychobiology, Federal University of São Paulo, São Paulo, Brazil
e Neuroimaging Research Section, Center for Addiction and Mental Health, Toronto, Canada

A R T I C L E   I N F O

Article history:
Received 15 February 2011
Received in revised form 26 May 2011
Accepted 31 May 2011
Available online 12 June 2011

Keywords:
Spider neurotoxin
Tx2-6
Phoneutria nigriventer
Penile erection
c-Fos
Sodium channel

A B S T R A C T

Brain areas expressing c-fos messenger RNA were mapped by quantitative in situ hybridization after 1–2 h of intoxication with 10 μg/kg Tx2-6, a toxin obtained from the venom of the spider Phoneutria nigriventer. Relative to saline-treated controls, brains from toxin-treated animals showed pronounced c-fos activation in many brain areas, including the supraoptic nucleus, the paraventricular nucleus of the hypothalamus, the motor nucleus of the vagus, area postrema, paraventricular and paratenial nuclei of the thalamus, locus coeruleus, central amygdaloid nucleus and the bed nucleus of the stria terminalis. The paraventricular hypothalamus and the bed nucleus of the stria terminalis have been implicated in erectile function in other studies. A possible role for central NO is considered. Acute stress also activates many brain areas activated by Tx2-6 as well as with NO-stimulated Fos transcription. Brain areas that appear to be selectively activated by Tx2-6, include the paratenial and paraventricular thalamic nuclei, the bed nucleus of the stria terminalis and the area postrema and the dorsal motor n. of vagus in the medulla. However, direct injections of different doses of the toxin into the paraventricular hypothalamic n. failed to induce penile erection, arguing against CNS involvement in this particular effect.

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1. Introduction

Human accidents involving the spider Phoneutria nigriventer are frequent in Brazil. Among the early signs of poisoning, excruciating localized pain, sweating, and nausea are commonly reported, while penile erection is rare but have been reported especially among young victims (Schenberg and Lima, 1966). Although priapism is a rare symptom in Phoneutria spider accidents, it can be consistently induced in mice under experimental conditions by injecting crude venom or the purified toxin Tx2-5 or Tx2-6. There is a clear dose-dependency and time-course and more important, it is the very first sign of intoxication so it can be induced in doses as low as to avoid other symptoms (described below). This strengthens the possibility of using Tx2-6 as a tool to manage erectile dysfunction or to investigate erectile mechanisms. Therefore, it is vital to understand the mechanisms by which Tx2-6 induces erection and the role of central and peripheral nervous system in this mechanism. On the other hand, in the event of a priapism in human patients, the knowledge of the mechanisms involved may also lead to a better treatment.
Activity-driven purification identified two priapism-inducing peptide toxins characterized by mass spectrometry and peptide sequencing (Edman’s degradation) (Troncone et al., 1998; Yonamine et al., 2004) and recognized to be identical to the Tx2-5 and Tx2-6 previously published (Rezende Junior et al., 1991). Other effects of intoxication by these toxins in mice include piloerection, tremors, intense salivation and, in the terminal stages of intoxication, a behavior that resembles clonic convulsions with characteristic movements of the forelimbs while standing on the hind limbs. However no signs of pain were observed when these purified toxins were injected intraperitoneally. Patch-clamp studies in frog neuromuscular junction using a semi-purified fraction containing the above toxins induced a delay in inactivation of sodium channels (Araujo et al., 1993).

We have demonstrated that iodinated Tx2-6 can penetrate the blood–brain barrier and thus potentially exert at least some of its effects via direct CNS stimulation (Yonamine et al., 2005). In the present investigation we mapped the brain areas showing increased c-fos transcription, a widely used marker of regional brain activation (Dragunow and Faull, 1989; Morgan and Curran, 1991), after systemic intoxication by Tx2-6 in doses that maximized the induction of penile erection. To further investigate whether the toxin induces penile erection by a central effect we injected different amounts of Tx2-6 directly into the paraventricular hypothalamic nucleus.

2. Material and methods

2.1. Toxin preparation

Spider venom purification was as described (Troncone et al., 1995; Yonamine et al., 2004) with modifications. Briefly, spider venom was obtained by electric milking, desiccated, resuspended in 2% (v/v) acetic acid, filtered and centrifuged to remove solids, and then applied to a Sephadex G50f chromatographic column. The fraction that produced the characteristic penile erection, salivation and death after i.p. injection was then lyophilized, resuspended in water and submitted to RP-HPLC using a TSK ODS 120-T Pharmacia column with linear gradient of trifluoroacetic acid (0.1% in water, v/v) and acetonitrile (90% in phosphoric acid, v/v); the gradient run from 10 to 90% of acetonitrile in 15 min. The active toxin showed as a single chromatographic peak. This active peak was further analyzed by mass spectrometry in a Perkin–Elmer Sciex API-III mass spectrometer by electrospray ionization. The sample was introduced by flow injection, with running solvent 50/50 ACN/H2O 0.1% HoAc, 1 mM NH4OAc.

2.2. Toxin injections

Ten male Swiss mice weighing 20–25 g were injected intraperitoneally with 1.0 μg/kg of Tx2-6 toxin (6 animals) or 0.1 ml of physiologic saline (4 animals). This dose was chosen based on previous dose-response studies in order to allow the animals to survive between 1 and 2 h and present full penile erections; lower doses led to incomplete erections. Signs of intoxication developed after approximately 15–20 min after injection. The first sign was penile erection, which was assessed by holding the animal and gently exposing the penis. Penile erections were observed in all animals injected with the toxin. Penile erection persisted until the death of the animal as a consequence of the intoxication, which occurred between 45 and 120 min after the injection. Control animals where handled as many times and identically as toxin-injected ones but no penile erection was observed; control animals were sacrificed by cervical dislocation 2 h after saline injection. Brains were quickly removed and frozen over dry ice, wrapped in aluminum foil and stored at −80 °C. Fifteen micrometers coronal brain sections were subsequently cut on a Jung-Reichert cryostat at −20 °C, mounted on polylysine-coated microscope slides (Sigma), briefly dried and stored at −80 °C until hybridization procedures.

2.3. In situ hybridization

A synthetic oligonucleotide complementary to bases 542 to 586 of the rat c-fos gene was used. The probe was labeled at the 3' end with 33P-alpha dATP (NEN Dupont, Boston, Mass). Slide mounted sections were first permeabilized with 0.3% Triton X-100, treated for 15 min in proteinase K at 37 °C, and fixed in 4% formaldehyde. Sections were then rinsed and pre-hybridized for 2 h at 37 °C in a solution containing, 6X SSC, 5X Denhardt’s solution, 200 μg/ml sheared salmon sperm DNA, 0.125M sodium pyrophosphate, 200 μg/ml yeast tRNA, 2 mM EDTA and 50% formamide. Sections were then hybridized for 18 h 42 °C in a solution similar to the one used for prehybridization, except for the addition of 20% dextran sulfate, 0.1 mg/ml polyadenylcylic acid, and the 33P-labeled c-fos oligo probe. Sections were then rinsed 3 × 15 min in 2× SSC at room temperature, 3 × 15 min in 2× SSC at 50 °C, and 1× SSC at 50 °C. They were then air dried and exposed to Hyperfilm-max film (Amersham) for 3 weeks in the presence of calibrated standards. Developed films were analyzed by computer-assisted densitometry using the MCID system (Imaging Research, St. Catharines, ON, CA) with a resolution of 8 bits/pixel. Anatomical regions were defined using the Franklin and Paxinos mouse brain atlas (Franklin and Paxinos, 1997). After films were developed, brain sections were stained with cresyl-violet to aid in the identification of anatomical boundaries.

2.4. Intracerebral toxin injection

Twenty three male Swiss mice weighting 25 g were employed in this experiment. Animals were anesthetized by xylazine/ketamine 12/80 mg/kg i.p. and positioned in a stereotaxic apparatus for the implantation of permanent guide cannulae in the right paraventricular hypothalamic nucleus (PVH) using the following coordinates in relation to bregma: 0.25 L, −0.94 AP and 3.6 V. The injection needle was 1 mm longer than the guide cannula. These coordinates were chosen after a series of pilot trials using methylene blue as a marker and cryostat sectioning to check for the injection site. Toxin or saline were injected in 3 μl volumes infused during 60 s with a needle attached to PE-10 tubing.
and a Hamilton syringe. Three animals were injected with saline for control purposes and 6 different concentrations of Tx2-6 were tested. Two animals were injected with 3 μg of toxin, six with 1.5 μg, three with 0.06 μg, six with 0.006 μg and three with 0.0006 μg. Animals were injected one at a time and immediately observed for behavioral signs and penile erections for up to 20 min.

3. Results

3.1. Toxin characterization

Mass spectrometry revealed that the peak of interest contained two different peptides. The major peak corresponded to a molecular mass of 5287 and the contaminant to a molecular mass of 6056, therefore a peptide. The proportion of these peptides was roughly estimated as 2:1. Trace amounts of two additional contaminants were also detected with molecular masses of 6127 and 6366. The predominant toxin showed characteristic peaks at m/z = 1058.2, 1322.6 and 1763.2. A toxin with the same pharmacological characteristics isolated from this venom and presenting a MW of 5291 (estimate obtained through Bio-Ion time of flight plasma desorption mass spectrometry) was fully sequenced (Cordeiro et al., 1992) and was named Tx2-6. Since the toxin used in the present study had the same N-terminal sequence we assume it to be Tx2-6. It is noteworthy that Tx2-6 eluted as a single HPLC peak and the contaminant was detected on the very sensitive process of MS. Other fractions of this batch obtained during the same purification and containing the single contaminant with MW = 6056 were devoid of effects when injected in mice. It has been shown by us and subsequently by others that Tx2-6 (and the isof orm Tx2-5) is the toxin involved in priapism. In the current experiments the symptom was observed during the intoxication therefore the contaminant would not have the same effect since it should be produced by other fractions too. The possible effects of the contaminant are further addressed in Discussion.

3.2. Fos activation in brain

Complete results obtained in this experiment are shown in Table 1. Relative to saline-treated controls, brains from toxin-treated animals showed pronounced c-fos activation in many areas, including the supraopti c nucleus (+286%), the paraventricular nucleus of the hypothalamus, the motor nucleus of the vagus (+201%), area postrema (+198%), paraventricular (+176%) and paratenial (+150%) nuclei of the thalamus, locus coeruleus (+146%), central amygdaloid nucleus (+133%) and the bed nucleus of the stria terminalis (+89%). Some of these regional effects are illustrated in Fig. 1.

3.3. Intracerebral toxin injection

Table 3 shows the behavior of each mouse after intracerebral injection of different doses of the toxin. Mice injected with the higher doses of Tx2-6 (3 and 1.5 μg) showed behavioral convulsions, ipsilateral and contralateral rotations, tremors, respiratory distress and died in less than 2 min. It is noteworthy that rigor mortis developed in less than 3 min, as is observed in mice injected intraperitoneally with this toxin. Mice injected with 0.06 and 0.006 typically presented contratralateral turning and convulsions immediately after injection and for a few seconds and died in about 20 min. Some animals had hypersalivation. Mice injected with the 0.0006 μg dose did not show behavioral signs of intoxication.

4. Discussion

The main hypothesis challenged in this investigation is whether Tx2-6 induces penile erection by selectively stimulating brain structures or by peripheral actions. Thus, one goal of this study was to determine the brain areas stimulated during intoxication by Tx2-6 and to compare the obtained c-fos pattern to known mappings of brain areas involved in penile erection or innervations of male genital organs. This comparison is presented in Table 2. Previous work described that pseudo rabies virus injected in rat penis was transported retrogradely and found in the nucleus paragigantocellularis, paraventricular hypothalamus, some raphe nuclei and Barrington’s nucleus, among other structures (Marson et al., 1993). These brain structures have also been implicated in erectile function in studies involving lesions or electrical stimulation (Holstege and Tan, 1987; Loewy et al., 1979; Marson and McKenna, 1990). The bed nucleus of the stria terminalis was identified as a key region in the control of non-contact erection in rats (Liu et al., 1997; Schmidt et al., 2000). The present observations of significant c-fos activation in the
paraventricular hypothalamus and the bed nucleus of the stria terminalis are thus consistent with a role for these structures in toxin-induced penile erection.

Previous studies tried to identify the venom factor responsible for penile erection. Crude fractions of *P. nigroventer* venom were found capable of relaxing rabbit cavernous strips “in vitro” (Lopes-Martins et al., 1994). Subsequently, a 17,000 Da polypeptide that induced this relaxation was isolated and partially sequenced (Rego et al., 1996) showing identity with another toxin Tx1 (8,000 Da) that had already been fully sequenced (Diniz et al., 1990) and for which cDNA encoding was described (Diniz et al., 1993). However, a fraction containing the toxin Tx1 called PhTx1 failed to induce penile erection after intracerebroventricular injections (Rezende Junior et al., 1991). However these authors described that a fraction of this venom containing the toxin Tx2-6 called PhTx-2 did induce penile erection when injected intracerebrally, in contrast to our results. We were able to induce priapism only by the intraperitoneal route. A possible explanation for this discrepancy is that in other studies the toxin may have leaked from the brain compartment reaching the bloodstream and then inducing priapism by a peripheral effect. In our laboratory purified Tx1 injected in identical conditions in mice (i.p.) failed to induce penile erection but induced all other symptoms seen with Tx2-5 and Tx2-6 injections. Finally, Cruz-Höfling and collaborators have described brain expression of the Fos protein after crude *P. nigroventer* venom i.v. injection as well as the expression of neural nitric oxide synthase (nNOS), this time in rats. Their results showed increased expression of the Fos protein in stress-related areas among others not detected in our experiments. Their studies investigated the effects of crude venom on the blood–brain barrier integrity and showed a relative and reversible breakdown as a result of systemic *Phoneutria* crude venom i.v. injections.

**Fig. 1.** Digitized images illustrating increased c-fos expression after Tx2-6 toxin administration at various levels of the mouse brain. Abbreviations: BnST: bed nucleus of the stria terminalis; PT: paratenial thalamus; PVN: paraventricular hypothalamus; SON: supraoptic nucleus; PVA: paraventricular thalamus, anterior division; CeM: central amygdaloid nucleus, medial division; AP: area postrema; 10: dorsal motor n. of Vagus. Tx2-6 sections in the leftmost column were used to generate the cresyl-violet images on the right most column.
humans, mice, guinea pigs and dogs but could not be induced in rats and rabbits (Schenberg and Lima, 1966).

It is conceivable that our observations involve nitric oxide synthesis. In a recent study we demonstrated that blockade of the neuronal nitric oxide synthase by 7-nitroindazole i.p. completely abolished the effects of Tx2-5, an isoform of toxin Tx2-6 studied here (Yonamine et al., 2004). These two toxins have identical pharmacological effects, both on sodium channels and in vivo (discussed below). We also demonstrated that iodinated Tx2-6 can penetrate the blood–brain barrier and thus potentially exert some effects directly on the CNS (Yonamine et al., 2005). In addition, intracerebroventricular injections of about 1 μg of a semi-purified fraction containing Tx2-6 induced all the symptoms including penile erection (Rezende Junior et al., 1991). Few studies described brain areas that respond to NO-donors with increased c-fos transcription. Fos positive areas in studies using i.c.v. injections of the NO-donor NOC-18 (Chikada et al., 2000) or subcutaneous nitroglycerin (Tassorelli and Joseph, 1995) were the bed nucleus of the stria terminalis, the paraventricular and paraventricular nuclei of the thalamus, the area postrema and dorsal motor nucleus of the vagus. These same areas were affected by Tx2-6 in our study. The dorsal motor nucleus of the vagus plays an important role in various visceral reflexes and its over stimulation may reflect an attempt to maintain internal balance disrupted by the toxin. The paraventricular and paratrigeminal thalamic nuclei stimulated by Tx2-6 seem to be part of a complex network of brain structures that control visceral awareness, together with the central amygdala, bed n. stria terminalis and accumbens, all of them involving nitric oxide to some extent (Van der Werf et al., 2002).

Another aspect to be considered is the extent to which the observed c-fos expression effects may reflect generalized stress associated with Tx2-6 intoxication. In a number of studies of c-fos activation following different types of acute stress, the paraventricular and supraoptic hypothalamic nuclei, the central amygdala and locus coerules have been found to be remarkably and consistently activated (Cullinan et al., 1995; Honkaniemi et al., 1992; Larsen and Mikkelsen, 1995; Li et al., 1996; Liu and Chen, 1994; 7-Liu et al., 1997; 8-Loewy et al., 1979; 9-Marson and Mckenna, 1990; 10-Marson et al., 1993; 11-Miyata et al., 1994; 12-Smith et al., 1995; 13-Vizuete et al., 1995; 14-Shehab et al. 1992).

Finally, since the proposed mechanism of action of Tx2-6 involves a delay in sodium channel inactivation (Araujo et al., 1993: Rizzi et al., 2007) and since the intoxication by the similar toxin Tx2-5 can be fully prevented by nNOS blockade (Yonamine et al., 2004), we are tempted to correlate these two observations. Indeed, sodium channels can be modulated by nitrosilation of its subunits by NO, as well as other ion channels (Li et al., 1998; Hammarstrom and Gage, 1999; Ahern et al., 2000; Renganathan et al., 2002). The question whether channel nitrosilation or direct toxin effects on channel gating is the primary effect of these toxins and others with similar properties, remain to be answered through specific experimentation.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>ECS14 PTZ14 Stress</th>
<th>Tx2-6 Brain areas involved in genital functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>3 3 +6 0</td>
<td></td>
</tr>
<tr>
<td>Olfactory cortex</td>
<td>4 4</td>
<td></td>
</tr>
<tr>
<td>Hippocampus, dentate gyrus</td>
<td>4 4</td>
<td>0</td>
</tr>
<tr>
<td>Bed n. stria terminalis</td>
<td>2 2 3 +7</td>
<td></td>
</tr>
<tr>
<td>Caudate-putamen</td>
<td>0 1</td>
<td>0</td>
</tr>
<tr>
<td>Amygdala</td>
<td>amygdalo-hippocampal n.</td>
<td>4 4 0</td>
</tr>
<tr>
<td>posteromedial cortical n.</td>
<td>4 4</td>
<td>0</td>
</tr>
<tr>
<td>basomedial n.</td>
<td>4 1</td>
<td>0</td>
</tr>
<tr>
<td>central n.</td>
<td>1 2 +3 3</td>
<td></td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>dorsomedial n.</td>
<td>4 1 +1 0</td>
</tr>
<tr>
<td>ventromedial n.</td>
<td>4 0</td>
<td>0</td>
</tr>
<tr>
<td>paraventricular n.</td>
<td>2 3 +1,2,5,11-13 4 +10</td>
<td></td>
</tr>
<tr>
<td>Supraoptic N.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thalamus, paraternal n.</td>
<td>2 2</td>
<td>4</td>
</tr>
<tr>
<td>Thalamus</td>
<td>2 2 +1 4</td>
<td></td>
</tr>
<tr>
<td>paraventricular n.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripeduncular n.</td>
<td>3 0</td>
<td>0</td>
</tr>
<tr>
<td>Periaqueductual grey a.</td>
<td>3 1 +1 0</td>
<td></td>
</tr>
<tr>
<td>Periaqueductal dorsal grey a.</td>
<td>3 0</td>
<td></td>
</tr>
<tr>
<td>Lateral parabrachial n.</td>
<td>3 2 +4 0</td>
<td></td>
</tr>
<tr>
<td>Locus coerules</td>
<td>1 2 +4 3</td>
<td></td>
</tr>
<tr>
<td>n. Solitary tract</td>
<td>2 3 +1,4</td>
<td></td>
</tr>
<tr>
<td>Lateral</td>
<td>1 1 +1 0</td>
<td>−2,7−10</td>
</tr>
<tr>
<td>paragigantocellular n.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS noradrenergic cell group</td>
<td></td>
<td>−10</td>
</tr>
<tr>
<td>Raphe magnus</td>
<td>+1 0</td>
<td>−10</td>
</tr>
<tr>
<td>Raphe pallidus</td>
<td>+1 0</td>
<td>−10</td>
</tr>
<tr>
<td>Parapyramidal ret. formation</td>
<td></td>
<td>−10</td>
</tr>
<tr>
<td>Vagus – dorsal motor n.</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Area postrema</td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>


a Rank ordering values from negligible or weak (0) to strongest (4) activation.
b Identified in lesion, stimulation and tract-tracing studies.
In summary, our results do not support CNS involvement in the pro-erectile action of Tx2-6. Although several brain areas seem to undergo strong stimulation during intoxication the specific areas involved are both related to penile erection and stress. On the other hand, the possibility that convulsions contribute to some of these effects seems unlikely. The c-fos results would be consistent with a more specific role for the bed nucleus of the stria terminalis, the paratenial and paraventricular nuclei of the thalamus, and the area postrema. The role of each of these structures in Tx2-6 induced erectile function could be ascertained by localized intracerebral microinfusions. Our experiments with direct injections onto the PVN suggests that this structure could be ruled out. At this point therefore, the hypothesis that this toxin induces penile erection by direct CNS actions should be considered with caution.

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Supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) to LRPT (94/1214-6) and Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq - No. 200538/95-0 to LRPT. D.C.H. was the recipient of a doctoral fellowship and K.G.R was the recipient of a M.Sc. fellowship from C.N.Pq. (Brazil).

Conflict of interest

The authors declare no conflict of interest regarding the content of this manuscript.

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