Neuroscience Letters 569 (2014) 121–125

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Effects of substance P and Sar-Met-SP, a NK1 agonist, in distinct amygdaloid nuclei on anxiety-like behavior in rats

Gabriel Shimizu Bassi[a,b], Milene Cristina de Carvalho[a,b,*], Marcus Lira Brandão[a,b]

[a] Instituto de Neurociências e Comportamento, Av. do Café, 2.450, 14050-220 Ribeirão Preto, SP, Brazil
[b] Laboratório de Neuropsicofarmacologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto (FFCLRP), Universidade de São Paulo (USP), Av. dos Bandeirantes, 3900, 14040-901 Ribeirão Preto, SP, Brazil

HIGHLIGHTS

- The role of NK1 receptors of the amygdala of rats was investigated in the EPM.
- Injection of SP or Sar into the CeA or MeA, but not the BLA, evoked anxiogenic-like effects.
- Anxiogenic-like effects of the SP in the CeA or MeA are mediated by NK1 receptors.

ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 7 March 2014
Accepted 27 March 2014

Keywords:
Amygdala
Substance P
NK-1 receptors
Elevated plus maze
Anxiety

ABSTRACT

The amygdala, together with the dorsal periaqueductal gray (dPAG), medial hypothalamus, and deep layers of the superior and inferior colliculi, constitutes the encephalic aversion system, which has been considered the main neural substrate for the organization of fear and anxiety. The basolateral nucleus of the amygdala (BLA) acts as a filter for aversive stimuli to higher structures while the central (CeA) and the medial (MeA) nuclei constitute the output for the autonomic and somatic components of the emotional reaction through major projections to the limbic and brainstem regions. Although some findings point to the distinct participation of the substance P (SP) and the NK1 receptors system in the different nuclei of the amygdala on the expression of emotional behaviors, it is not clear if this system modulates anxiety-like responses in the distinct nuclei of the amygdala as well as the dPAG. Thus, it was investigated if the injection of SP into the BLA, CeA, or MeA affects the expression of anxiety-like responses of rats submitted to the elevated plus-maze (EPM) test and, if the effects are mediated by NK1 receptors. The results showed that SP and Sar-Met-SP (NK1 receptor selective agonist) injected into the CeA and MeA, but not into the BLA, caused anxiogenic-like effects in the EPM. Altogether, the data indicates that the SP may mimic the effects of anxiogenic stimuli via NK1 receptor activation only in the CeA and MeA (amygdala's nuclei output) and may activate the neural mechanisms involved in the defensive reaction genesis. The SP/NK1 receptors system activation may be phasically involved in very specific aspects of anxiety behaviors.

1. Introduction

The organization of fear and anxiety-like behaviors has been related to an encephalic aversion system composed of the amygdala, medial hypothalamus, dorsal periaqueductal gray (dPAG), and deep layers of the superior and inferior colliculi [4,16]. Several reports point to the amygdaloid complex as an important functional link in the neurocircuitry responsible for the integration of aversive states in the central nervous system [21,22]. Indeed, it has been shown that the amygdala processes the stimulus input from the environment and, depending on the type of the threat, engages with the neural substrate of fear in the dPAG [15,18,21,38] to which it sends direct projections [7,26]. The mechanisms

Abbreviations: BLA, basolateral nucleus of the amygdala; CeA, central nucleus of the amygdala; dPAG, dorsal periaqueductal gray; EPM, elevated plus maze; MeA, medial nucleus of the amygdala; NK, neurokinin receptors; PAG, periaqueductal gray; Sal, saline; Sar, Sar-Met-SP; SP, substance P.

* Corresponding author at: Laboratório de Neuropsicofarmacologia – Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, USP, Av. Bandeirantes, 3.900, Ribeirão Preto, SP, 14090-901, Brazil. Tel.: +55 16 3602 3837; fax: +55 16 3602 4830.
E-mail addresses: mccarva@usp.br, mccarvalhorp@gmail.com (M.C. de Carvalho).

http://dx.doi.org/10.1016/j.neulet.2014.03.065
0304-3940/

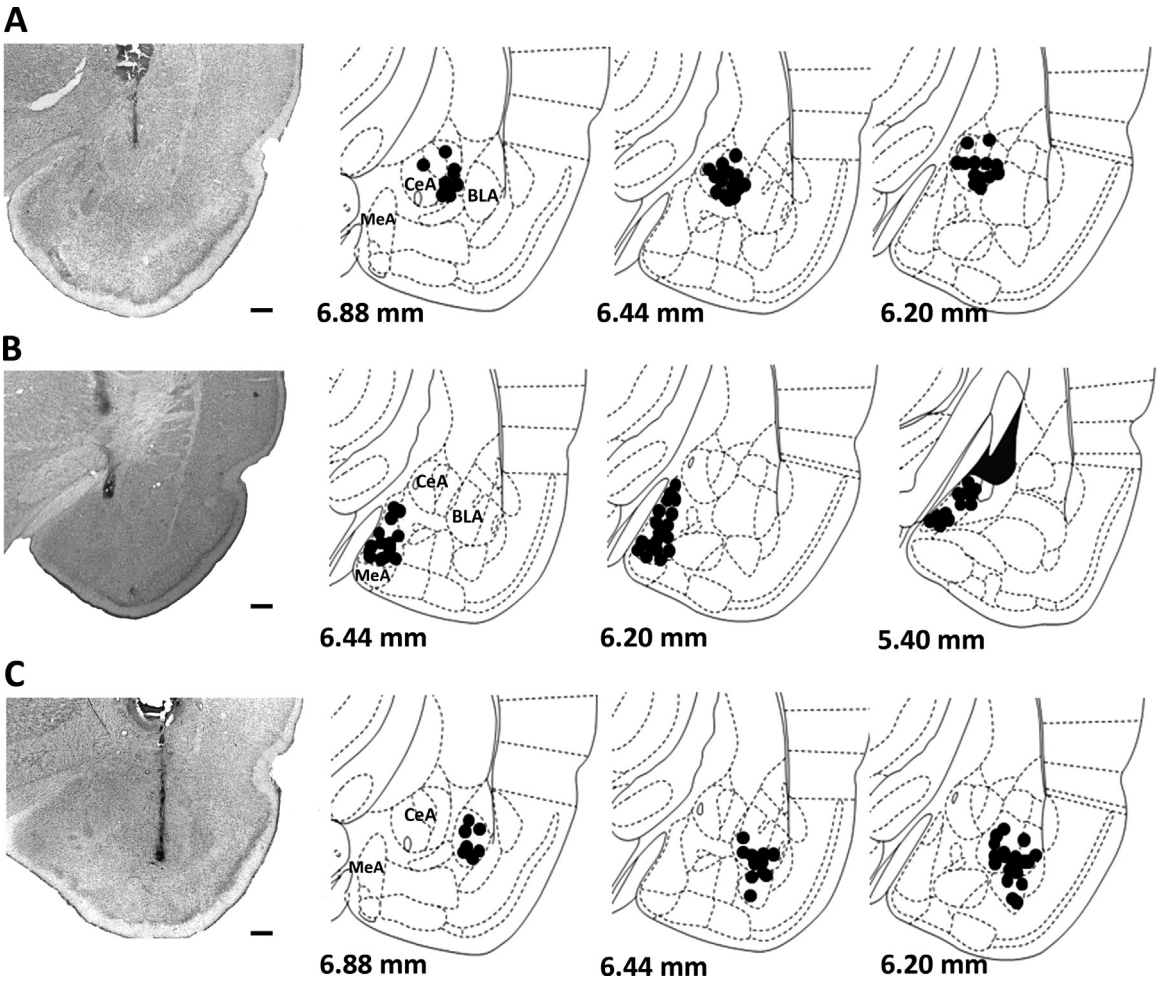

Fig. 1. (A) Representative photomicrographs of microinjections into the central (A), medial (B) and basolateral (C) nuclei of the amygdala and outline of the injection sites on cross-sections from Paxinos and Watson [27]. The distance from the interaural line is indicated below each section. The number of sites indicated in the figures is less than the number of injected rats because of several overlaps. Scale bars represent 500 μm in all photographs. CeA: central nucleus of the amygdala, MeA: medial nucleus of the amygdala and BLA: basolateral nucleus of the amygdala.

underlying modulation of anxiety-like responses depend on which amygdaloid nucleus is activated [2,11]. The basolateral amygdala (BLA) is predominantly involved in the filtering of aversive stimuli while the central (CeA) as well as the medial nucleus (MeA) constitute the output for the autonomic and somatic components of the defensive reaction via major projections to the hypothalamus and brainstem regions [5,32]. The excitability of these output neurons is regulated by a tonic inhibitory influence from the BLA [24].

Substance P (SP) is a neurokinin that is widely distributed in the brain and has been shown to modulate stress and anxiety-linked behaviors [13,14,20]. A large number of peptidergic neurons in the amygdala, including the neurokininergic neurons, project to the PAG [17] where microinjection of SP elicits defensive behaviors [9,10]. Three NK receptors have been identified so far: NK-1, NK-2, and NK-3. Despite the fact that SP binds to all receptor types, it shows much higher affinity for NK-1 receptors [8,29], which are heterogeneously distributed in the amygdaloid complex [30,31,35]. Activation of SP/NK1 receptors in the different amygdaloid nuclei has been shown to modulate the expression of learned and innate defensive responses of rats [3,6,36,38]. SP injected into the CeA or MeA, but not in the BLA, also produced anxiogenic-like effects in the elevated plus-maze (EPM), an effect that was blocked in the presence of a NK1 antagonist [6,12]. We have now extended these findings by investigating the effect of activating NK1 receptors using the highly selective NK1 agonist Sar-Met-SP and compared its effectiveness in different amygdaloid nuclei [23].

2. Materials and methods

Male Wistar rats weighing 150–180 g were obtained from the breeding facility of the University of São Paulo at Ribeirão Preto. Animals were housed in a temperature-controlled (22 ± 1 °C) room and maintained on a 12 h light/12 h dark cycle with lights on at 7:00 a.m. Rats were housed in groups of five per cage (40 cm × 33 cm × 18 cm) and given free access to food and water. The experiments were performed in accordance with the Brazilian Society of Neuroscience and Behavior Guidelines for the Care and Use of Laboratory Animals. The procedures were approved by the Committee on Animal Research and Ethics (CEUA) of the University of Sao Paulo (No. 09.1.84.54.7).

The animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) with the upper incisor bar at 3.3 mm below the interaural line. A unilateral stainless steel guide cannula (12 mm, 24 gauge) aimed at the right BLA, CeA or MeA was implanted in each animal. With the interaural line as the reference point for each plane, the following coordinates were used: for BLA – antero-posterior (AP) 6.1 mm, medio-lateral (ML) 4.7 mm, and dorso-ventral (DV) 5.3 mm; for CeA – AP 6.1 mm, ML 4.2 mm, and DV 4.9 mm and for MeA – AP 6.2 mm, ML 3.2 mm, and DV 6.0 mm [27].

Experiments began 5 days after guide cannula implantations. The rats were subjected to the EPM test as described in detail

in previous studies [6,28]. Briefly, the rats received intra-BLA, -CeA, or -MeA microinjections of SP (35 pmol/0.2 μl; Sigma-USA) or the NK-1 agonist Sar-Met-SP (Sar; 50 and 100 pmol/0.2 μl; Sigma-USA). Both drugs were dissolved in saline (Sal) and the doses used were selected based on previous publications from our laboratory [1,10]. In order to minimize the spread of the volume of the injections, we used a glass micropipette for microinjections, according to a technique described elsewhere [25,33]. In brief, micropipettes were made of a fused silica capillary (o.d. 150 μm, i.d. 75 μm; Cluzeau Info Lab, France). To prevent cannula breaks, the capillary was fixed in a device made with needles of 0.60 × 25 mm and 1.00 × 25 mm (Becton–Dickinson). The fused silica capillary protruded 3.0 mm beyond the guide-cannula to reach the BLA, CeA or MeA. The micropipette was linked to a 5 μl Hamilton syringe by means of polyethylene tubing (PE-10; Becton–Dickinson, Franklin Lakes, NJ) connected to a microinfusion apparatus (Harvard, USA). A volume of 0.2 μl was injected over 60 s. Following the end of the injections, the microinjection pipettes were held inside the brain for a further 60 s to maximize diffusion of the drug away from the tip. Immediately after SP or 5 min after the Sar-Met-SP microinjections, the rat was gently placed in the central area of the EPM with the nose facing one of the closed arms and its behavior was observed for 5 min. The exploratory behavior (conventional EPM measures, i.e., number of entries and time spent in the arms) was recorded by a video camera interfaced with a monitor and a VCR in an adjacent room for later analysis (Noldus software, Amsterdam, The Netherlands). The luminosity at the level of the open arms of the EPM was 20 lx.

Upon completion of the experiments, the animals were deeply anesthetized with urethane and perfused intracardially with 0.9% Sal followed by 10% formalin solution. Three hours later, the brains were immersed in 30% sucrose. Seven days later, the brains were frozen and serial brain sections (60 μm) were cut using a cryostat (Leica, Wetzlar, Germany), thaw-mounted on gelatinized slides and stained with cresyl violet in order to localize the sites of injection with reference to Paxinos and Watson [27].

3. Results

Data are expressed as mean + SEM. One-way analysis of variance (ANOVA) was used to assess the effects of SP and Sar-Met-SP microinjections into the BLA, CeA or MeA. Fisher's least significant difference post hoc comparisons were performed when significant overall *F*-values were obtained in the ANOVA ($p < 0.05$).

Photomicrographs of representative microinjections sites and the location of the cannula tips and in the BLA, CeA, or MeA are shown in Fig. 1A–C. Following injection of Sar-Met-SP into CeA and MeA, one-way ANOVA showed significant differences in the frequency of entries in the open arms ($F3,29 = 11.32$ and $F3,35 = 14.28$, respectively; $p < 0.05$ in all cases) and in the percentages of time spent in the open arms/total ($F3,29 = 6.13$ and $F3,35 = 93.60$, respectively; $p < 0.05$ in all cases). Post hoc comparisons revealed that injections of both SP and Sar-Met-SP into the CeA and MeA reduced the frequency of entries in the open arms as well as the percentages of time spent in these arms/total in relation to the control group (Fig. 2A and C, respectively). There was no significant differences in the frequency of closed arm entries: $F3,29 = 0.23$; $p > 0.05$ for the CeA and $F3,35 = 1.44$; $p > 0.05$ for the MeA (Fig. 2B). In contrast, the statistical analysis showed no significant differences in the measures evaluated in the EPM test (frequency of entries in the open and closed arms ($F3,31 = 0.39$ and 0.46; $p > 0.05$, respectively) and in the percentages of time spent in the open arms/total ($F3,31 = 0.45$; $p > 0.05$) (Fig. 2A–C) after microinjections of SP or Sar-Met-SP into the BLA. Due to the similarity of the effects of both doses of Sar-Met-SP on the behavior of rats in the EPM, we chose to illustrate only the effects of the dose of 50 pmol in Fig. 2A–C.

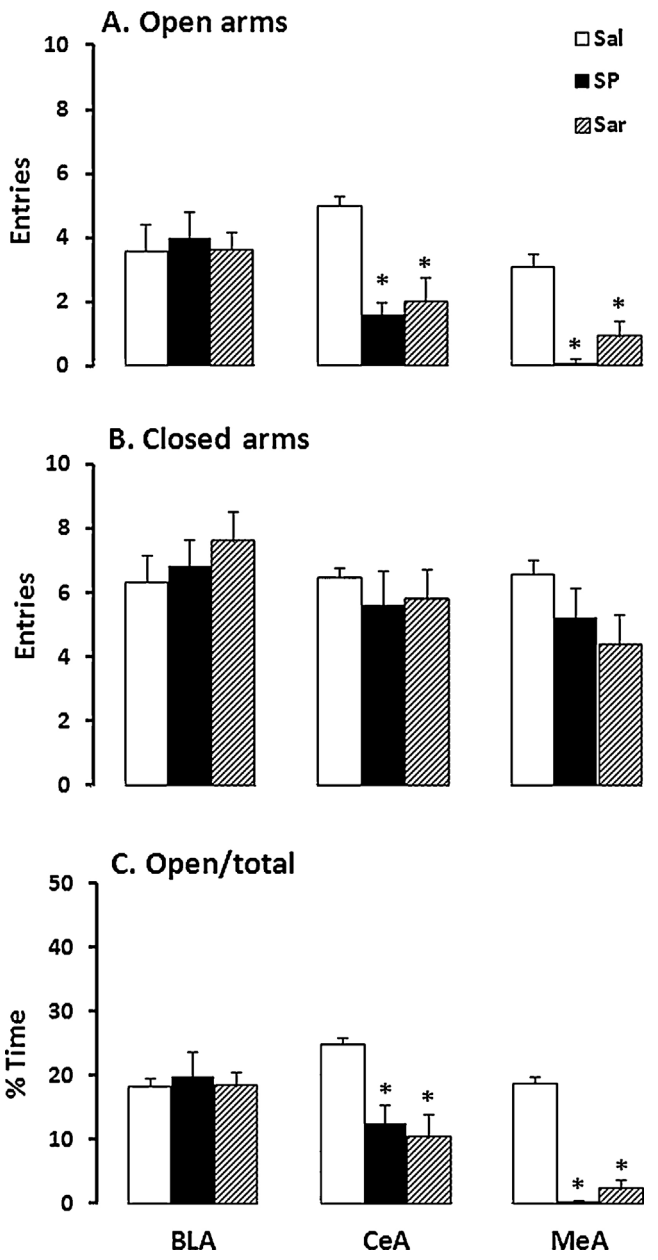

Fig. 2. Effects of SP (35 pmol/0.2 μL) or Sat-Met-SP (Sar, 50 pmol/0.2 μL) injected into the basolateral (BLA), central (CeA), or medial (MeA) nuclei of the amygdala on rat behavior in the elevated plus maze. Each bar represents mean + SEM of frequency of entries in the open and closed arms (A and B, respectively) and % of time spent in the open arms compared with total entries (C). *$p < 0.05$ compared with control (Sal). BLA (Sal: $n = 7$; SP: $n = 10$; Sar: $n = 8$), CeA (Sal: $n = 9$; SP: $n = 10$; Sar: $n = 7$), and MeA (Sal: $n = 11$; SP: $n = 9$; Sar: $n = 11$).

4. Discussion

The present study showed that SP injected into the CeA or MeA, but not into the BLA, caused anxiogenic-like effects in the EPM, in agreement with previous findings [6,12] suggesting that SP produces defensive behaviors in this point of the encephalic aversion system as well as in the dPAG and hypothalamus [6,9,10,12,34]. These effects of SP were reproduced by microinjection of Sar-Met-SP, a neurokinin agonist with high selectivity for the NK1 receptor in brain tissue [23]. Thus, NK1 receptor activation in the CeA and also in the MeA is able to modulate the aversiveness associated with the open space and height that the EPM represents to the animal [28]. These effects cannot be attributed to motor alterations since

the treatments did not change the number of entries in the closed arms of the EPM.

The dose of SP used in the present study was chosen based on a dose we had found previously to be effective in the PAG [10]. It is however, higher than the doses that have since been reported by us and by others to be effective in the amygdala [6,12]. Indeed, when injected into the amygdala bilaterally SP was effective at much lower doses than used here [12]. Like other peptides, often produces a dose-related effect that is commonly represented by an inverted U-shaped dose response relationship [9,12,19,35]. We have shown previously that responsiveness to SP after unilateral injection into the amygdala is essentially constant over the dose range of 10–100 pmol [6]. Therefore, we believe that the effects of SP seen in the present study represent a physiological action of the peptide. However, we cannot rule out the possibility that at the dose used (35 pmol), SP may not be selective for the NK1 receptor subtype.

The NK1 receptor-mediated effects of Sar-Met-SP were localized to the CeA and MeA. Both nuclei are the main output for the autonomic and somatic components of the emotional reactions via their projections to the hypothalamus and brainstem regions [5,32]. Interestingly, in the present study the administration of SP or Sar-Met-SP into the BLA did not produce any effect on behavior in the EPM, in agreement with a previous reported [6]. In contrast, other studies have suggested an involvement of NK1 receptors in the BLA on the expression of emotional responses. However, in those studies the rats were submitted to a different type of stressful procedure than the EPM [3,36,38]. Thus, the kind of aversion elicited in different models may be relevant. In the EPM, unlike immobilization stress, maternal separation, and fear potentiated startle, the animals have the choice to avoid the aversive environment. In other words, the aversive stimulus is completely avoidable depending on the free decision of the rat. It is possible that the SP/NK1 receptor system of the BLA does not participate in this type of decision-making process, even though this amygdaloid nucleus tonically inhibits the excitability of the output neurons of CeA and MeA [24] and is linked with the dPAG [37]. On the other hand, SP in the CeA or MeA may modulate the defensive mechanisms by acting on the NK1 receptors only when they are called into play by the presence of aversive stimuli, i.e., this mediation is phasic [6,12]. Thus, there may be differential participation of the NK1 receptor system in different amygdaloid nuclei depending on the context of a stress, i.e., whether some control over the environment is given to the rat on the control of this response.

Acknowledgements

This work was supported by CAPES project grant 23038.027765/2009-10; Aux-PE-PNPD 2193/2009 to M.C.C. and M.L.B. GSB holds a master scholarship from CNPq (Proc. n° 133713/2010-5). M.C.C. was funded by a CAPES postdoctoral award (Proc. n° 23038.027765). The authors are indebted to Dr. T.A. Lovick for reviewing this manuscript.

References

[1] G.S. Bassi, M.J. Nobre, J.E. de Araujo, M.L. Brandao, Anxiogenic effects of activation of NK-1 receptors of the dorsal periaqueductal gray as assessed by the elevated plus-maze, ultrasound vocalizations and tail-flick tests, Neuropeptides 41 (2007) 365–374.
[2] D.C. Blanchard, R.J. Blanchard, Innate and conditioned reactions to threat in rats with amygdaloid lesions, J. Comp. Physiol. Psychol. 81 (1972) 281–290.
[3] S. Boyce, D. Smith, E. Carlson, L. Hewson, M. Rigby, R. O'Donnell, T. Harrison, N.M. Rupniak, Intra-amygdala injection of the substance P [NK(1) receptor] antagonist L-760735 inhibits neonatal vocalisations in guinea-pigs, Neuropharmacology 41 (2001) 130–137.
[4] M.L. Brandao, V.Z. Anseloni, J.E. Pandossio, J.E. De Araujo, V.M. Castilho, Neurochemical mechanisms of the defensive behavior in the dorsal midbrain, Neurosci. Biobehav. Rev. 23 (1999) 863–875.
[5] N.S. Canteras, R.B. Simerly, L.W. Swanson, Organization of projections from the medial nucleus of the amygdala: a PHAL study in the rat, J. Comp. Neurol. 360 (1995) 213–245.
[6] M.C. Carvalho, J.M. Santos, G.S. Bassi, M.L. Brandao, Participation of NK1 receptors of the amygdala on the processing of different types of fear, Neurobiol. Learn. Mem. 102 (2013) 20–27.
[7] M.D. Cassell, D.J. Wright, Topography of projections from the medial prefrontal cortex to the amygdala in the rat, Brain Res. Bull. 17 (1986) 321–333.
[8] T.V. Dam, E. Escher, R. Quirion, Evidence for the existence of three classes of neurokinin receptors in brain. Differential ontogeny of neurokinin-1, neurokinin-2 and neurokinin-3 binding sites in rat cerebral cortex, Brain Res. 453 (1988) 372–376.
[9] J.E. de Araujo, J.P. Huston, M.L. Brandao, Aversive effects of the C-fragment of Substance P in the dorsal periaqueductal gray matter. Experimental brain research, Exp. Hirnforsch. 123 (1998) 84–89.
[10] J.E. De Araujo, R.C. Silva, J.P. Huston, M.L. Brandao, Anxiogenic effects of substance P and its 7-11C terminal, but not the 1-7N terminal, injected into the dorsal periaqueductal gray, Peptides 20 (1999) 1437–1443.
[11] R.A. Dielenberg, G.E. Hunt, I.S. McGregor, 'When a rat smells a cat': the distribution of Fos immunoreactivity in rat brain following exposure to a predatory odor, Neuroscience 104 (2001) 1085–1097.
[12] K. Ebner, N.M. Rupniak, A. Saria, N. Singewald, Substance P in the medial amygdala: emotional stress-sensitive release and modulation of anxiety-related behavior in rats, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 4280–4285.
[13] K. Ebner, S.B. Sartori, N. Singewald, Tachykinin receptors as therapeutic targets in stress-related disorders, Curr. Pharm. Des. 15 (2009) 1647–1674.
[14] K. Ebner, N. Singewald, The role of substance P in stress and anxiety responses, Amino Acids 31 (2006) 251–272.
[15] M.S. Fanselow, The Midbrain Periaqueductal Gray as a Coordinator of Action in Response to Fear and Anxiety, Plenum Publishing Corp., New York, 1991, pp. 151–173.
[16] F.G. Graeff, Brain Defense Systems and Anxiety, vol. 3, Elsevier Science Publishers, Amsterdam, 1990, pp. 307–354.
[17] T.S. Gray, D.J. Magnuson, Peptide immunoreactive neurons in the amygdala and the bed nucleus of the stria terminalis project to the midbrain central gray in the rat, Peptides 13 (1992) 451–460.
[18] C.T. Gross, N.S. Canteras, The many paths to fear, Nat. Rev. Neurosci. 13 (2012) 651–658.
[19] J.P. Huston, M.S. Oitzl, The relationship between reinforcement and memory: parallels in the rewarding and mnemonic effects of the neuropeptide substance P, Neurosci. Biobehav. Rev. 13 (1989) 171–180.
[20] V. Kormos, B. Gaszner, Role of neuropeptides in anxiety, stress, and depression: from animals to humans, Neuropeptides 47 (2013) 401–419.
[21] J.E. Ledoux, The amygdala: contributions to fear and stress, Sem. Neurosci. 6 (1994) 231–237.
[22] J.E. LeDoux, The emotional brain, fear, and the amygdala, Cell. Mol. Neurobiol. 23 (2003) 727–738.
[23] R. Lew, D.P. Geraghty, G. Drapeau, D. Regoli, E. Burcher, Binding characteristics of [125I]Bolton-Hunter [Sar9,Met(O2)11]substance P, a new selective radioligand for the NK1 receptor, Eur. J. Pharmacol. 184 (1990) 97–108.
[24] L. Nitecka, Y. Ben-Ari, Distribution of GABA-like immunoreactivity in the rat amygdaloid complex, J. Comp. Neurol. 266 (1987) 45–55.
[25] M.J. Nobre, M.L. Brandao, Analysis of freezing behavior and ultrasonic vocalization in response to foot-shocks, ultrasound signals and GABAergic inhibition in the inferior colliculus: effects of muscimol and midazolam, Eur. Neuropsychopharmacol. 14 (2004) 45–52.
[26] O.P. Ottersen, Afferent connections to the amygdaloid complex of the rat with some observations in the cat. III. Afferents from the lower brain stem, J. Comp. Neurol. 202 (1981) 335–356.
[27] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Elsevier Inc, New York, 2005, pp. 1–367.
[28] S. Pellow, P. Chopin, S.E. File, M. Briley, Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat, J. Neurosci. Methods 14 (1985) 149–167.
[29] D. Regoli, G. Drapeau, S. Dion, P. D'Orleans-Juste, Pharmacological receptors for substance P and neurokinins, Life Sci. 40 (1987) 109–117.
[30] G.W. Roberts, P.L. Woodhams, J.M. Polak, T.J. Crow, Distribution of neuropeptides in the limbic system of the rat: the amygdaloid complex, Neuroscience 7 (1982) 99–131.
[31] M. Saffroy, J.C. Beaujouan, Y. Torrens, J. Besseyre, L. Bergstrom, J. Glowinski, Localization of tachykinin binding sites (NK1, NK2, NK3 ligands) in the rat brain, Peptides 9 (1988) 227–241.
[32] P. Sah, E.S. Faber, M. Lopez De Armentia, J. Power, The amygdaloid complex: anatomy and physiology, Physiol. Rev. 83 (2003) 803–834.
[33] J.M. Santos, C.E. Macedo, M.L. Brandao, Gabaergic mechanisms of hypothalamic nuclei in the expression of conditioned fear, Neurobiol. Learn. Mem. 90 (2008) 560–568.
[34] M.B. Shaikh, A. Steinberg, A. Siegel, Evidence that substance P is utilized in medial amygdaloid facilitation of defensive rage behavior in the cat, Brain Res. 625 (1993) 283–294.
[35] N. Singewald, G.G. Chicchi, C.C. Thurner, K.L. Tsao, M. Spetea, H. Schmidhammer, H.K. Sreepathi, F. Ferraguti, G.M. Singewald, K. Ebner, Modulation of basal and

stress-induced amygdaloid substance P release by the potent and selective NK1 receptor antagonist L-822429, J. Neurochem. 106 (2008) 2476–2488.
[36] D.W. Smith, L. Hewson, P. Fuller, A.R. Williams, A. Wheeldon, N.M. Rupniak, The substance P antagonist L-760,735 inhibits stress-induced NK(1) receptor internalisation in the basolateral amygdala, Brain Res. 848 (1999) 90–95.
[37] J.M. Zanoveli, C. Ferreira-Netto, M.L. Brandao, Conditioned place aversion organized in the dorsal periaqueductal gray recruits the laterodorsal nucleus of the thalamus and the basolateral amygdala, Exp. Neurol. 208 (2007) 127–136.
[38] Z. Zhao, Y. Yang, D.L. Walker, M. Davis, Effects of substance P in the amygdala, ventromedial hypothalamus, and periaqueductal gray on fear-potentiated startle, Neuropsychopharmacology 34 (2009) 331–340.