Neuroscience Letters 474 (2010) 154–157

Contents lists available at ScienceDirect

Neuroscience Letters

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

Intra-amygdala injections of neuropeptide S block fear-potentiated startle

Markus Fendt[a,*], Stefan Imobersteg[a], Hugo Bürki[a], Kevin H. McAllister[a], Andreas W. Sailer[b]

[a] Novartis Institutes of BioMedical Research, Neuropsychiatry, GPCR Expertise Program, Basel, Switzerland
[b] Novartis Institutes of BioMedical Research, Developmental and Molecular Pathways, GPCR Expertise Program, Basel, Switzerland

ARTICLE INFO

Article history:
Received 18 January 2010
Received in revised form 19 February 2010
Accepted 9 March 2010

Keywords:
Amygdala
Anxiety
Conditioned fear
Locomotor activity
Mice

ABSTRACT

Injections of neuropeptide S (NPS) into the lateral ventricle induce a strong hyperactivity. Since most behavioral paradigms are dependent of spontaneous locomotor activity, this makes it difficult to interpret the role of NPS in such paradigms. The aim of the present experiment was to investigate the effects of NPS in fear-potentiated startle, a behavioral fear paradigm which we believe is less sensitive to general changes in locomotor activity. Furthermore, NPS was directly injected into the amygdala, the central site of the neural fear circuitry. Our data shows that intra-amygdala NPS injections dose-dependently block the expression of conditioned fear and that this effect is independent of NPS effects on locomotor activity. This strongly supports a crucial role of amygdaloid NPS in conditioned fear.

Neuropeptide S (NPS) and its cognate receptor (NPSR) are a recently identified G protein-coupled receptor (GPCR)/ligand pair which have been linked to multiple physiologically important processes [12,17]. First isolated from rat whole brain extracts, Sato and colleagues demonstrated that a 20 amino acid peptide with a terminal serine designated NPS can modulate the activity of the NPSR, formerly called TGR23-2, GPR154, and GPRA (WO2002031145 Japanese, EP1329508A1, English). Using this information, Xu et al. [22] were the first to delineate a role of NPS in the central nervous system. A detailed expression analysis of the ligand and its receptor uncovered the expression of NPSR throughout the central nervous system, including brain centers that regulate fear and arousal [21,22].

Central administration of NPS strongly induced locomotor hyperactivity, suppressed all stages of sleep, reduced food intake, enhanced self-administration of drugs of abuse, and had anxiolytic-like effects in several animal models of anxiety. A detailed characterization of the NPS system is therefore crucial for the understanding of its physiological role in health and disease. For example, two recent studies investigated the role of NPS in conditioned fear in detail [7,11]: On the cellular level, NPS modulated activity patterns in the basolateral amygdala via the endopiriform cortex. Furthermore, glutamatergic transmission to intercalated GABAergic neurons in the amygdala was increased. Both mechanisms may lead to a net inhibition of the amygdala. Thus local injections of NPS into the amygdala or into the endopiriform cortex were found to have anxiolytic-like effects on the expression of conditioned fear and to facilitate extinction of conditioned fear, respectively.

A major criticism of all prior studies which have investigated the role of NPS in fear and anxiety is that behavioral readouts were used which are not only sensitive to anxiolytic-like treatments but also to treatments affecting locomotor activity. Both intracerebroventricular and intra-amygdala injections of NPS induce robust hyperactivity (present study, [7,18,22]). Such hyperactivity intrinsically leads to a higher probability to visit anxiety-inducing areas, as well as to a reduction of freezing (which is measured as a cessation of body movement). In the end, these interactions may imply a high risk of false-positive findings, i.e. unspecific effects on behavioral readouts of fear and anxiety [9,20].

We believe that the fear-potentiated startle paradigm is less sensitive to general changes in locomotor activity than traditional anxiety models like elevated plus maze. This view is supported by experiments showing that treatments which change locomotor activity but have no anxiolytic-like effects do not affect fear-potentiated (e.g., [6]). As such, possible effects of intra-amygdala NPS injections on locomotor behavior should not interfere with possible effects on the expression of fear-potentiated startle, and provide a relatively unbiased estimate of the true effects of NPS in conditioned fear. The aim the present study was therefore to investigate the role of intra-amygdala NPS in the fear-potentiated startle paradigm.

Experimentally naive male mice (DBA1/J, supplied by Janvier, Le Genest Saint Isle, France) aged 2–3 months were used. The animals were housed in groups of 2–4 in a humidity- and temperature-controlled room under a 12/12 h day-night cycle with lights on

* Corresponding author at: Novartis Institutes of BioMedical Research, WSJ-386.3.28, Forum 1, Novartis Campus, CH-4056 Basel, Switzerland.
Tel.: +41 61 3241042; fax: +41 61 3244502.
E-mail address: markus.fendt@novartis.com (M. Fendt).

0304-3940/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2010.03.028

at 07:00 am. Water and food were available ad libitum. The animal cages were equipped with plastic nest boxes, wooden chew blocks, and nesting material. Animal experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and approved by the Basel City Cantonal Veterinary Authority.

Mice were anesthetized with ketamine/xylazine (110 mg/kg, 10:1, i.p.) and placed into a stereotaxic frame. The skull was exposed and stainless steel guide cannulae (diameter: 0.35 mm; length: 6 mm) were bilaterally implanted to the amygdala using the following coordinates [14]: 1.5 mm caudal from Bregma, ±3.5 mm lateral from Bregma, −3.7 mm ventral from dura. The guide cannulae were fixed to the skull with dental cement and 2–3 anchoring screws. To prevent post-surgery pain, the analgesic buprenorphin (0.01 mg/kg, i.p.) was given twice per day on the first 2 days following surgery. Behavioral tests started following full recovery (5–6 days after surgery).

Fear-potentiated startle: A startle system with eight chambers (35 cm × 35 cm × 38 cm) was used (SR-LAB, San Diego Instruments, San Diego, CA). Each chamber contained a small animal enclosure made of transparent Plexiglas cylinder with 4 cm inner diameter and 10 cm inner length. Movements of the animals were detected by motion-sensitive transducers mounted underneath the cylinders. For data acquisition the output signal of the transducers was digitized (sampling rate: 1 kHz) and stored on a computer. Stored responses were expressed in arbitrary units.

For fear conditioning, electric foot shocks and light stimuli were used. The light stimulus was presented to the animals with 20 W bulbs (ca. 700 lx) mounted to the back of the test chambers. Scrambled foot shocks were administered by a floor grid (seven parallel bars, 8 mm apart and 4 mm diameter) and had an intensity of 0.6 mA and a duration of 1 s. White background noise (50 dB SPL) and the acoustic noise stimuli (96 dB SPL) were generated by high-frequency loudspeakers mounted in the center of the ceiling of the test chambers.

Locomotor activity: A computerized motility measurement system was used (Moti 4.25, TSE Systems, Bad Homburg, Germany). This system automatically measures locomotor activity in transparent boxes (20 cm × 32 cm × 17 cm) by counting the interruptions of horizontal infrared beams spaced 5.7–8.4 cm apart in a frame set at the cage-floor level of the boxes.

Mouse NPS used in this study was custom synthesized by NeoMPS (Strasbourg, France) and dissolved in sterile saline (NaCl 0.9%). For the intracranial injections, the mice were gently restrained by the experimenter, the injectors with a diameter of 0.15 mm (connected to Hamilton syringes by tubes) were introduced into the guide cannulae, and the animals were released in a cage. A total volume of 0.3 μl solution was then injected at a flow rate of 0.1 μl/min, controlled by a microinfusion pump (CMA100, CMA, Stockholm, Sweden). The injector was removed after additional 60 s. The mice were then returned into their home cages. After additional 10 min, the animals were put into the startle system.

Sixty DBA1 mice with implanted intra-amygdala cannulae were first tested for baseline acoustic startle response to 10 startle stimuli and then allocated into two treatment groups with similar mean baseline startle magnitudes. On the following 2 days, the animals were conditioned using a fear-potentiated startle protocol. On each day, after a habituation period of 5 min, they received 10 pairings of 30 s light stimulus and a foot shock which was presented in the last second of the light stimulus. The mean intertrial interval was 140 s (range: 100–180 s).

On the 4th day, vehicle, 0.01, 0.1 or 1 nmol NPS/side (0, 0.02, 0.22, and 2.2 μg/side) was injected bilaterally into the amygdala (n = 15/group). Then the animals were put into the startle devices and after an acclimatization period of 5 min, 12 startle stimuli of 96 dB SPL were administered, half of them alone, the other half were preceded by the conditioned light stimulus (in a pseudorandomized order). Inter-stimulus interval was 120 s and no shocks were administered on this retention test day.

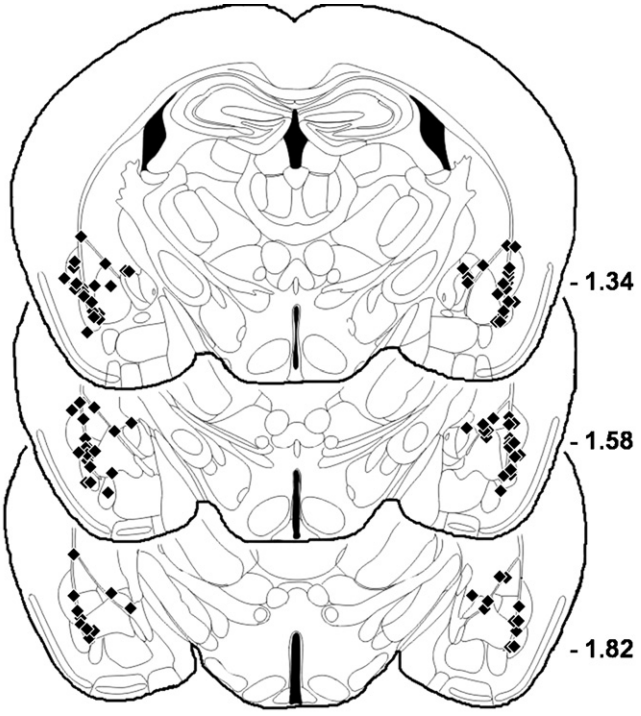

Fig. 1. Frontal sections of the amygdala depicting the injection sites of the different experiments into the amygdala. *Abbreviations*: BLA, basolateral amygdala; CA, central amygdala; LA, lateral amygdala.

Immediately after the fear-potentiated startle test, the animals were put into the motility boxes, and their spontaneous locomotor activity was tested for 30 min.

Immediately after the final behavior test, all mice were euthanized. For the verification of the amygdala cannulae, the brains were removed and immersion-fixed with 4% formaldehyde 30% sucrose. Frontal sections (100 μm) were cut on a freezing microtome and counterstained with cresyl violet. The injection sites were localized and the extent of tissue lesions due to cannulation was examined under a light microscope. The injection sites were confirmed by comparison with plates taken from a mouse brain atlas [14].

All reported statistical tests were performed using the program SYSTAT (SPSS Inc., version 12). For analysis of the behavioral data, analyses of variance (ANOVA; if appropriate with repeated measures) and post hoc Dunnett's tests were used.

Histological analysis confirmed bilateral injections into the amygdala in 44 animals (Fig. 1). These mice were used for the analysis of the effects of intra-amygdala NPS injections on fear-potentiated startle. The remaining animals had either unilateral or misplaced injections or lesions of the amygdala caused by the cannulae and were excluded from further analysis.

The fear-potentiated startle data (Fig. 2A) were analyzed with an ANOVA using "treatment" (different NPS doses) as between subject factor and "trial type" (startle-alone vs. CS-startle) as within subject factor. The factor trial type had significant effects ($F_{1,40}$ = 16.43, p < 0.001) indicating successful fear conditioning. NPS injections into the amygdala dose-dependently decreased fear-potentiated startle demonstrated by a significant interaction treatment x trial type ($F_{3,40}$ = 3.99, p = 0.014). The factor treatment had no main effects ($F_{3,40}$ = 0.63, p = 0.60). In a second analysis, we used only the

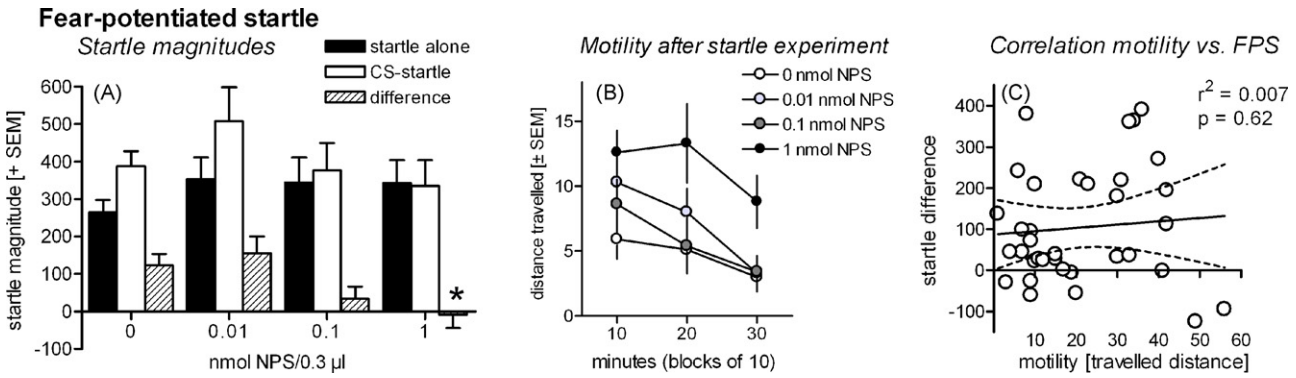

Fig. 2. (A) Bar diagram showing the effects of NPS injections into the amygdala on the expression of fear-potentiated startle. The bars represent the mean startle magnitudes (+SEM) after tone alone (black bars) and light-tone trials (white bars), as well as the difference scores (hatched bars). *$p < 0.05$, Dunnett's test after significant main effects in ANOVA. (B) Diagram depicting the locomotor activity (distance travelled) of the animals tested immediately after the fear-potentiated startle test. (C) Correlation between fear potentiation (*Y*-axis) and motility (*X*-axis). Each dot represents an individual animal, the line is the linear fit, and the dashed line is the 95% confidence interval.

startle magnitudes of the first startle-alone trial and CS-startle trial to check whether the NPS effects were already present at the beginning of the experiment. This analysis revealed a strong reduction of fear-potentiated startle already at the first trial (interaction treatment x trial type: $F_{3,40} = 3.66$, $p = 0.02$, trial type: $F_{1,40} = 5.29$, $p = 0.03$, treatment: $F_{3,40} = 0.38$, $p = 0.77$). With both analyses, 1 nmol NPS was the only effective dose (post hoc Dunnett's test, comparisons with vehicle treated animals: p's < 0.05).

Directly after the test on fear-potentiated startle, the animals were put in the motility setup to measure potential effects of intra-amygdala NPS injections on spontaneous locomotor activity. An ANOVA revealed significant effects on the travelled distance ($F_{3,40} = 3.41$, $p = 0.03$). Again, 1 nmol NPS was the effective dose (post hoc Dunnett's test: $p < 0.05$). We then checked whether or not, on an individual level, the increase in motility was correlated with the effects on fear-potentiated startle. This was clearly not the case (linear correlation analysis: $r^2 = 0.007$, $p = 0.62$). A similar analysis using the percent fear-potentiation scores similarly showed no correlation between fear-potentiated startle and locomotor activity ($r^2 = 0.04$, $p = 0.27$).

Fourteen animals in the present experiment were excluded from final analysis due to lesions caused by the cannulae ($n = 4$) or misplaced injections ($n = 10$, perirhinal cortex, caudate putamen, internal capsule). Only three of these animals with misplaced injections received 1 nmol NPS. However, all these animals showed fear-potentiated startle (means ± SEM: tone alone trials: 326 ± 141, CS-tone trials: 556 ± 105, difference: 230 ± 90). In addition, no increase in locomotor activity animals with misplaced was observed in these animals with misplaced 1 nmol NPS injections (18.8 ± 7.8; vehicle: 14.0 ± 3.8; intra-amygdala 1 nmol NPS: 34.8 ± 6.3).

The aim of the present study was to investigate the role of intra-amygdala NPS injection on the expression of fear-potentiated startle. Fear-potentiated startle was chosen as a behavioral readout for conditioned fear since we believe that it is less sensitive to general changes in locomotor activity. In previous studies, anxiolytic-like effects of both intracerebroventricular and intra-amygdala NPS injections were demonstrated in behavioral models of fear and anxiety. However, the readouts used in these studies are also sensitive to general changes in locomotor activity such as sedation or hyperactivity. This is critical since NPS administration induces a pronounced locomotor hyperactivity [18,22].

In general, there are two strategies to avoid potential interference between the behavioral readout of interest (here: fear behavior) and side-effects (here: hyperactivity): First, the use of local injections directly into the neural fear system, and, second, a behavioral model with a readout which we believe is less sensitive hyperactivity. The present study represents a combination of these two strategies, and with regard to the readout of interest, clearly demonstrated that injections of NPS into the amygdala dose-dependently decreased the expression of fear-potentiated startle. This clearly supports the previously published studies demonstrating anxiolytic-like effects of NPS in different behavioral models of innate anxiety and conditioned fear [7,11].

However, most of these aforementioned studies used intracerebroventricular NPS injections which are known to induce a pronounced hyperactivity. In contrast, Jüngling et al. [7] and Meis et al. [11] used local NPS injections, either into the lateral/basolateral amygdala or into the endopiriform cortex. Both of these authors stated that their local injections had no effects on locomotor activity. However, whereas the first of these two studies used a relatively low number of animals in the respective experiment (n's = 6–9), the second study based their interpretation on a readout that showed high variability (total arm entries in the elevated plus maze; SEM was ca. 2/3 of the mean). Both lead to a low statistical power. The present study, with groups sizes of 9–11 and much less variability in the primary readout (SEM = ca. 1/4 of mean), showed a robust and significant increase in locomotor activity in the open field after intra-amygdala NPS injections.

There are different possible explanations of this effect on locomotor activity. First, this increase in locomotor activity may represent an anxiolytic-like effect. The open field was novel for the animals, and in such situations, there is a conflict between exploratory drive and neophobia [16]. Reducing neophobia by intra-amygdala NPS would increase exploratory behavior. A second explanation could be that NPS diffused from the injection side within the amygdala to other brain sides which are involved in modulating locomotor behavior. In the present study, we injected 0.3 µl per side, and, also with our optimized infusion methods, such a volume will diffuse ca. 0.5–1.0 mm away from the injection site [1,4,10], i.e. not only the whole amygdala but very probably also neighboring brain structures which might be involved in modulating locomotor activity (e.g., caudate putamen) are flooded by the NPS solution.

In our study, to determine whether or not the NPS effect on fear-potentiated startle was independent of the effects on locomotor activity, we tested whether these two behaviors were correlated. This was clearly not the case, which (1) demonstrates that the anxiolytic and locomotor activity-stimulating effects of NPS are independent, and (2) in turn suggests that the locomotor activity effects of intra-amygdala NPS observed in the present study were probably due to the spread of NPS into neighboring brain areas involved in modulation of locomotor activity and not due to anxiolytic-like effects.

Most of our injection sites were localized within the central, lateral and basolateral part of the amygdala. However, as stated above, each of these injections will probably flood the whole amygdala, as well as neighboring brain areas. Electrophysiological studies demonstrated that GABAergic interneurons in the medial cluster of the paracapsular intercalated cell masses (localized between lateral and central amygdala), as well as projection neurons within the basolateral amygdala and the endopiriform cortex are sensitive to NPS administration. All these neuron types are involved in the modulation of amygdala's activity and thereby in the modulation of conditioned fear responses [5,7,11,15] and could potentially mediate the fear reduction observed in the present study.

In summary, the present study shows that NPS within the amygdala plays an important role in the expression of conditioned fear. We demonstrated clearly, for the first time, that the fear-reducing effects of NPS are independent of NPS effects on locomotor activity.

References

[1] T.A. Allen, N.S. Narayanan, D.B. Kholodar-Smith, Y. Zhao, M. Laubach, T.H. Brown, Imaging the spread of reversible brain inactivations using fluorescent muscimol, J. Neurosci. Methods 171 (2008) 30–38.
[4] J.-M. Edeline, B. Hars, E. Hennevin, N. Cotillon, Muscimol diffusion after intracerebral microinjections: a reevaluation based on electrophysiological and autoradiographic quantifications, Neurobiol. Learn. Mem. 78 (2002) 100–124.
[5] I. Ehrlich, Y. Humeau, F. Grenier, S. Ciocchi, C. Herry, A. Luthi, Amygdala inhibitory circuits and the control of fear memory, Neuron 62 (2009) 757–771.
[6] R.J.E. Joordens, T.H. Hijzen, B. Olivier, The anxiolytic effect on the fear-potentiated startle is not due to a non-specific disruption, Life Sci. 63 (1998) 2227–2232.
[7] K. Jüngling, T. Seidenbecher, L. Sosulina, J. Lesting, S. Sangha, S.D. Clark, N. Okamura, D.M. Duangdao, Y.L. Xu, R.K. Reinscheid, H.C. Pape, Neuropeptide S-mediated control of fear expression and extinction: role of intercalated GABAergic neurons in the amygdala, Neuron 59 (2008) 298–310.
[9] C.M. Markham, M. Yang, R.J. Blanchard, D.C. Blanchard, Effects of D-amphetamine on defensive behaviors related to fear and anxiety, Pharmacol. Biochem. Behav. 83 (2006) 490–499.
[10] J.H. Martin, Autoradiographic estimation of the extent of reversible inactivation produced by microinjection of lidocaine and muscimol in the rat, Neurosci. Lett. 127 (1991) 160–164.
[11] S. Meis, J.R. Bergado-Acosta, Y. Yanagawa, K. Obata, O. Stork, T. Munsch, Identification of a neuropeptide S responsive circuitry shaping amygdala activity via the endopiriform nucleus, PLoS ONE 3 (2008) e2695.
[12] N. Okamura, R.K. Reinscheid, Neuropeptide S: a novel modulator of stress and arousal, Stress 10 (2007) 221–226.
[14] G. Paxinos, K.B.J. Franklin, The Mouse Brain in Stereotaxic Coordinates, San Diego, Academic Press, 2001.
[15] G.J. Quirk, E. Likhtik, J.G. Pelletier, D. Pare, Stimulation of medial prefrontal cortex decreases the responsiveness of central amygdala output neurons, J. Neurosci. 23 (2003) 8800–8807.
[16] A. Ramos, Animal models of anxiety: do I need multiple tests? Trends Pharmacol. Sci. 29 (2008) 493–498.
[17] R.K. Reinscheid, Neuropeptide S: anatomy, pharmacology, genetics and physiological functions, Results Probl. Cell Differ. 46 (2008) 145–158.
[18] A. Rizzi, R. Vergura, G. Marzola, C. Ruzza, R. Guerrini, S. Salvadori, D. Regoli, G. Calo, Neuropeptide S is a stimulatory anxiolytic agent: a behavioural study in mice, Br. J. Pharmacol. 154 (2008) 471–479.
[20] G. Vitale, M. Filaferro, V. Ruggieri, S. Pennella, C. Frigeri, A. Rizzi, R. Guerrini, G. Calo, Anxiolytic-like effect of neuropeptide S in the rat defensive burying, Peptides 29 (2008) 2286–2291.
[21] Y.L. Xu, C.M. Gall, V.R. Jackson, O. Civelli, R.K. Reinscheid, Distribution of neuropeptide S receptor mRNA and neurochemical characteristics of neuropeptide S-expressing neurons in the rat brain, J. Comp. Neurol. 500 (2007) 84–102.
[22] Y.L. Xu, R.K. Reinscheid, S. Huitron-Resendiz, S.D. Clark, Z.W. Wang, S.H. Lin, F.A. Brucher, J.A. Zeng, N.K. Ly, S.J. Henriksen, L.C. Lecea, O. Civelli, Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects, Neuron 43 (2004) 487–497.