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Peripherally applied neuropeptide SF is equally algogenic in wild type and ASIC3−/− mice

Yevgen K. Yudin *, Zinayida A. Tamarova, Oleg A. Krishtal

Bogomoletz Institute of Physiology, Bogomoletz Str. 4, 01024 Kiev, Ukraine

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Abstract

RFa-related peptides play a significant role in the processing of pain in the CNS of mammals. Recently it has been found that, when applied subcutaneously, these peptides elicit a powerful algogenic effect. The question arises whether this peripheral effect can be connected with the ability of RFa-related peptides to decrease the rate of desensitization of acid sensing ionic channels (ASICs) expressed in primary sensory neurons. We have addressed this question by comparing the effects of neuropeptide SF (NPSF), mammalian RFa peptide, in ASIC3−/− and wild-type C57BL/6J mice. Knockout of ASIC3 gene results in the changes in some of the behavioral parameters. However, subcutaneous injections of the NPSF into the *n.saphenous* innervation area result in a clearly nociceptive behavior in both strains of mice. There is no significant difference in the total time of licking of injected paw in the ASIC3−/− (194 ± 22 s) and C57BL/6J (227 ± 25 s) animals. Thus peripheral algogenic effects of NPSF cannot be explained only in terms of their action on the ASIC3 channels and involves some other, still unidentified mechanism.

Keywords: RFamide-related peptides; ASICs; Pain

1. Introduction

The history of RFa-related peptides has started with the identification of physiologically active tetrapeptide FMRFa in molluscs (Price and Greenberg, 1977). A remarkable number of RFa-related peptides have been isolated since then. At least five different genes for RFa related peptides exist in mammals. They encode neuropeptide SF (NPSF), neuropeptide FF (NPFF), neuropeptide AF (NPAF) and recently discovered new peptides (Hinuma et al., 2000; Fukusumi et al., 2001). In mammals, RFa-related peptides participate in the modulation of opiate function (Tang et al., 1984; Malin et al., 1990), in cardiovascular regulation (Panula et al., 1996) and in the pain perception in CNS. Depending on the route of administration, RFa peptides induce opposite effects on the modulation of pain. When injected intracerebroventricularly, FMRFa or NPFF demonstrate antiopioid activity in rodents and reduce morphine induced analgesia (Roumy and Zajac, 1998; Malin et al., 1990; Tang et al., 1984). Intrathecally administrated NPFF produces long lasting antinociception (Gouarderes et al., 1993) and potentiates antinociceptive effects of morphine (Kontinen and Kalso, 1995). In the isolated DRG neurons RFa peptides slow-down the desensitization of ASICs, which presumably play role in nociception and protonsensitivity (Askwith et al., 2000). It has been demonstrated that NPSF, like other RFa-peptides, decreases the desensitisation rate and/or the sustained phase of current through homomeric ASIC3 channels without affecting its peak value (Deval et al., 2003).

RFa-related peptides serve as ligands for G protein coupled receptors that activate second messengers pathway in the sensory neurons (Elshourbagy et al., 2000; Bonini et al., 2000). NPFF has been found in small- and medium-sized DRG neurons (Allard et al., 1999) associated with small Aδ and C afferents fibers. The release of this peptide during inflammation (Kontinen et al., 1997; Vilim et al., 1999; Ferrarese et al., 1986) prompts that it may be effective not only in the CNS and spinal cord but also at the periphery.

Recently we have obtained the data confirming the idea of possible involvement of RFa-related peptides in the peripheral nociception. When examined in the skin-nerve preparation FMRFa and other RFa-related peptides induce strong excitation in the majority of the nociceptive C-fibers (Yudin et al., 2004).

* Corresponding author. Tel.: +380 44 2532466; fax: +380 44 2562590.
E-mail address: yy@biph.kiev.ua (Y.K. Yudin).

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Amiloride, which is the channel blocker of ASICs, inhibited this effect only in about half of the RFa-sensitive fibers. These data cannot either confirm or exclude the involvement of ASICs in the RFa-peptide mediated excitation of C-fibers. Specifically, ASIC3 channels that are characteristic for DRG neurons are the major targets for the action of RFa-peptides (Xie et al., 2003). However, the sustained current through the ASIC3 channel is not inhibited by amiloride (de Weille et al., 1998; Babinski et al., 1999). ASIC3 knockout mice display enhanced pain behavior to high intensity nociceptive stimuli regardless of their modality (Chen et al., 2002) and acid-induced hyperalgesia (Sluka et al., 2003). Increased ASIC3 expression in DRG during inflammation (Voilley et al., 2001; Yiangou et al., 2001) implies the involvement of these channels in allodynia and hyperalgesia. The question arises: are ASIC3 involved in the peripheral effects of RFa peptides? We have addressed this problem by studying the reactions of ASIC3 knockout mice to the endogenous RFa-related peptide of mammals, NPSF.

2. Methods

2.1. Animals

Two groups of mice have been used in these experiments: C57Bl/6J line, wild type mice (WT) and the same line without functional ASIC3 gene, knockout mice (KO). Male mice, 2–3 months old, were used for behavioral experiments. Each mouse was used in one experiment only. Weight of the animals was 20–30 g. Mice were housed in a temperature-controlled space and kept at a 12 h dark–light cycle. Animals had free access to food and water. Before the experiments they stayed for adaptation in the individual cages minimum 24 h. All the experiments were performed near the middle of the light period to reduce circadian effects on the nociceptive sensitivity (Kavaliers and Hirst, 1983).

2.2. Drugs and administration routes

The injections of peptide were made subcutaneously into the dorsal surface of the left hind paw of the animals that were kept in the movement restriction chamber. The volume of 10 μl per 10 g of the animals weight was injected via a microsyringe with a 27 gauge needle. NPSF were dissolved in the 0.9% isotonic solution of NaCl to a final concentration of 2 mM. Equal volume of 0.9% NaCl solution was used in the control experiments. The injections were made immediately prior to the beginning of the experiments.

2.3. Behavioral experiments

The animals representing two genome types (WT as the control and KO) were divided into six groups. Two groups of mice of each genome type ($n = 12$ per group) were used to evaluate normal behavior. Two groups of mice ($n = 10$ per group) received injection of 2 mM solution of NPSF. The last two groups of animals ($n = 10$ per group) were injected with 0.9% isotonic solution of the NaCl. After injection the animals immediately were returned to their cages to evaluate the behavior parameters. Five parameters of the behavior were registered for 60 min: duration of grooming, sleeping, running (non-pain parameters) and duration and frequency of licking of the injected paw (pain parameters). Sleeping was determined as the period of the animals immobility with closed eyes, the time counting was started after 10 s of this period. Frequency of licking was determined as the number of separate licks during the period of observations. Running was determined as the period of sharp, brisking movements, which the animals performed after a period of patience. All the experiments were performed in accordance with the ethical guidelines for investigation of the experimental pain in the conscious animals and guidelines of the Institutional Animal Care and Use Committee.

2.4. Statistical analysis

The obtained data were analyzed by one-way ANOVA followed by t-Student tests. The level of significance was set at $P < 0.05$. Results are presented as mean ± S.E.M.

3. Results

Comparative behavioral analysis of WT and KO mice in normal conditions revealed a difference in some measured parameters (Table 1). Thus, duration of grooming was 1.5 times shorter in KO mice ($P < 0.05$), while the total time of running in the cage was 2.5 shorter ($P < 0.01$) in the WT animals. The number of lickings in KO animals was also 1.75 times higher ($P < 0.05$). These data imply that the knockout of the ASIC3 gene results in the changes in normal behavior of mice.

Subcutaneous injection of isotonic 0.9% NaCl solution into the left hind paw did not produce any significant changes in the behavior of both groups of mice (Table 2). Administration of NPSF solution produced significant changes in the behavior in both groups of mice (WT and KO, Table 2) compare to the control injection. We have found significant differences in all the measured pain and non-pain behavior parameters except grooming in WT group of mice after injection of NPSF. The F-values ($F_{2,29}$) of the one-way ANOVA was 60.92 ($P < 0.001$) for the duration of licking of the injected paw and 36.67 ($P < 0.001$) for the number of licking. For the non-pain parameters F-values ($F_{2,29}$) were 5.21 ($P < 0.05$) for sleeping and 7.51 ($P < 0.01$) for running. NPSF injection induced a dramatic increase in the total time of licking of the injected paw (6.8 times, $P < 0.001$, see Fig. 1). The number of licking was also increased by the factor of 3.4 ($P < 0.001$), see Fig. 2. The sleeping period was 2.4 times shorter after NPSF injection

Table 1
Behavior parameters for 60 min observation in normal condition

Parameter	Normal	
	C57BL/6J	ASIC3−/−
Duration of licking of the left paw (s)	23.8 ± 4.89	38.9 ± 10.12 ($t = 1.34$)
Number of licking of the left paw (n)	5.3 ± 0.59	9.32 ± 1.27* ($t = 2.87$)
Grooming (s)	695.58 ± 84.38	442.92 ± 58.95* ($t = -2.45$)
Sleeping (s)	775 ± 174.59	537.67 ± 135.03 ($t = 1.07$)
Running (s)	9.83 ± 2.42	24.5 ± 3.9** ($t = 3.19$)

* $P < 0.05$.
** $P < 0.01$ compared with C57BL/6J (otherwise not significant).

Table 2
Behavior parameters for 60 min observation after subcutaneous injection of 0.9% NaCl or 2 mM NPSF in left hindlimb

Parameters	NaCl		NPSF	
	C57BL/6J	ASIC3−/−	C57BL/6J	ASIC3−/−
Duration of licking of the left paw (s)	28.5 ± 4.51	59.3 ± 9.75* ($t = 2.86$)	194.2 ± 21.86	227.3 ± 25.16 ($t = 0.99$)
Number of licking of the left paw (n)	8.2 ± 1.52	14.4 ± 1.62* ($t = 2.79$)	28.4 ± 3.32	47.2 ± 3.53*** ($t = 3.87$)
Grooming (s)	826.8 ± 97.63	730.6 ± 46.22 ($t = 0.89$)	801.9 ± 95.87	631.8 ± 84.54 ($t = 1.33$)
Sleeping (s)	479.5 ± 99.04	488.7 ± 105.6 ($t = 0.06$)	190.5 ± 57.62	81.5 ± 42.84 ($t = 1.51$)
Running (s)	5.1 ± 1.28	19.9 ± 5.02** ($t = 2.85$)	19.8 ± 3.76	31.4 ± 8.52 ($t = 1.24$)

* $P < 0.05$ compared with C57BL/6J (otherwise not significant).
** $P < 0.01$ compared with C57BL/6J (otherwise not significant).
*** $P < 0.001$ compared with C57BL/6J (otherwise not significant).

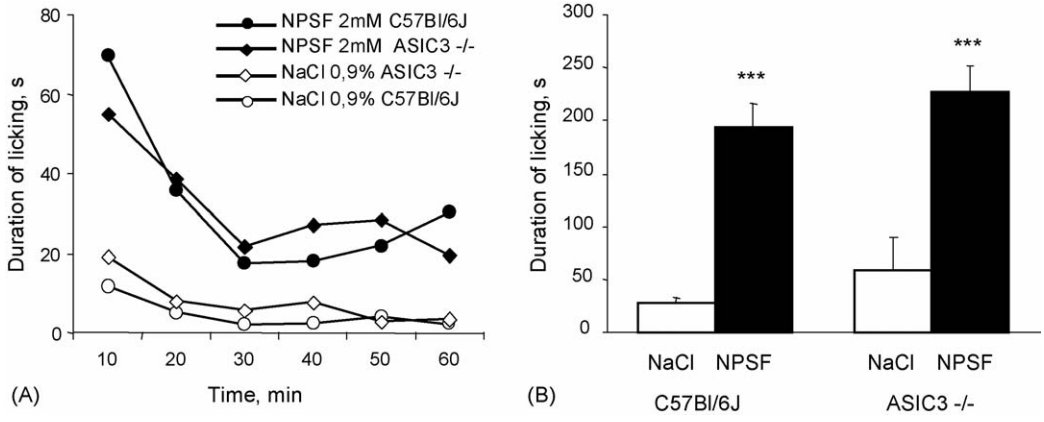

Fig. 1. Subcutaneous injections of NPSF induce nociceptive behavior in WT and KO mice, compare to the control injection of 0.9 % NaCl. (A) Time course of pain behavior reaction (licking of the staggered paw) caused by NPSF. Duration of licking was measured during each successive 10 min period after injection of a peptide. (B) Cumulative data for 60 min of observation. Bars represent mean ± S.E.M. ($n = 10$; ***$P < 0.001$, significance of a difference from the effect of NaCl injection).

($P < 0.01$) and the running period was 3.9 times longer ($P < 0.05$) as compared to the NaCl injected mice (Fig. 3). These data indicate that subcutaneously administered NPSF, like other previously studied RFa peptides (Yudin et al., 2004), is powerfully algogenic.

Similar strong reactions to NPSF administration were observed in the KO mice. The F-values ($F_{2,29}$) of the one-way ANOVA were 45.31 ($P < 0.001$) for licking duration of injected paw and 90.1 ($P < 0.001$) for the number of licking. For non-pain parameters the F-values ($F_{2,29}$) were 5.3 ($P < 0.05$) for sleeping and 5.32 ($P < 0.001$) for grooming. KO animals were licking the injected paw 3.8 times longer after NPSF injection ($P < 0.001$), see Fig. 1. The number of licking was increased by the factor of 3.2 ($P < 0.001$), see Fig. 2. The period of sleep was reduced six-fold ($P < 0.01$), (Fig. 3). These results indicate that subcutaneous administration of the NPSF induces strong changes in the

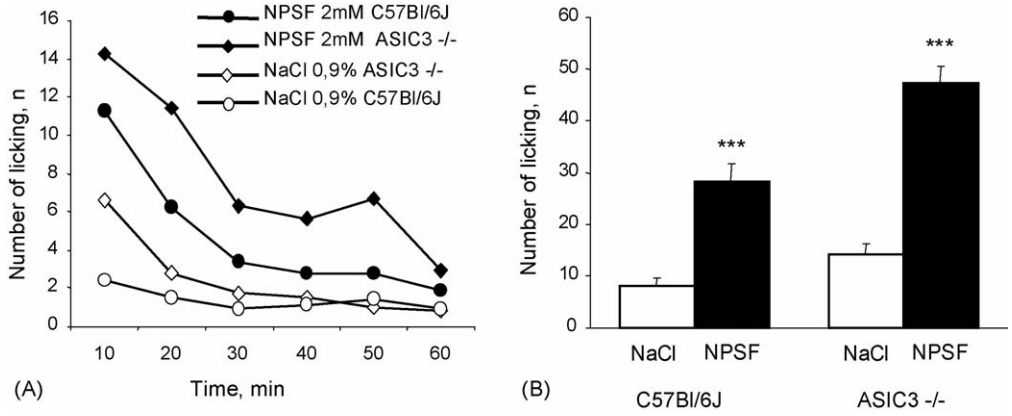

Fig. 2. Subcutaneous injections of NPSF induce nociceptive behavior in WT and KO mice, compare to the control injection of 0.9% NaCl. (A) Time course of pain behavior reaction (number of licking of the injected paw) caused by NPSF. Parameter duration was measured during each successive 10-min period after injection of a peptide. (B) Cumulative data for 60 min of observation. Bars represent mean ± S.E.M. ($n = 10$; ***$P < 0.001$, significance of a difference from the effect of NaCl injection).

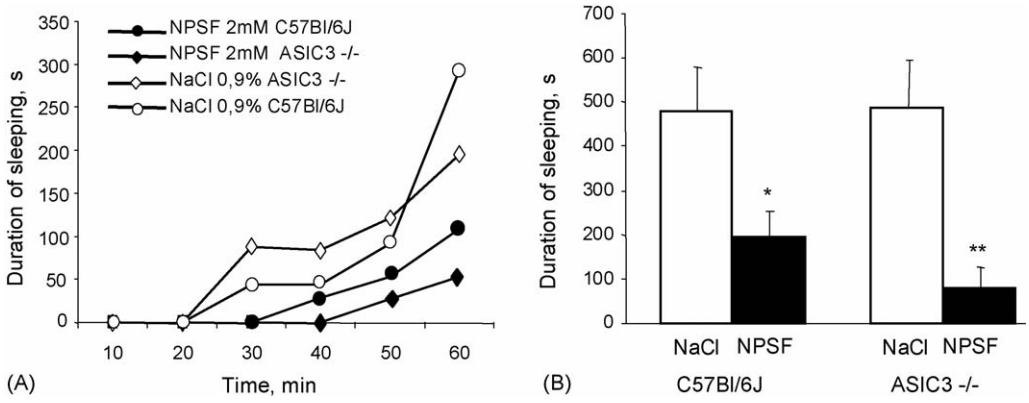

Fig. 3. Subcutaneous injections of NPSF reduce duration of sleeping for WT and KO mice, compare to the control injection of 0.9% NaCl. (A) Time course of changing in sleeping duration caused by NPSF. Duration of sleeping parameter was measured during each successive 10-min period after injection of a peptide. (B) Cumulative data for 60 min of observation. Bars represent mean ± S.E.M. ($n = 10$; $^{*}P < 0.05$; $^{**}P < 0.01$, significance of a difference from the effect of NaCl injection).

behavior of both C57BL/6J and ASIC3−/− groups of animals. In summary, we did not observe any difference in the effects of NPSF on both genotypes of the animals.

4. Discussion

Our experiments demonstrate that the disruption of ASIC3 gene induces changes in the normal behavior of mice. The KO mice are more anxious as compared to the WT animals. However, the pain parameters of their behavior (at least those studied here) seem to remain unaltered. In previous in-vivo experiments on KO mice it has been demonstrated that ASIC3 play a role in modulation of the responses to various moderate- and high-intensity pain stimuli regardless of their modality (Chen et al., 2002). In fact, the sensitivity to acid injection and to the thermal and mechanical pain was increased in KO animals. The intraperitoneal acid-induced writhing test revealed that KO mice were more sensitive to acid: they showed more writhings and had earlier reaction as compared to WT animals. Other group of investigators, using intradermal injected acid (pH 3) test, demonstrated that paw licking, as the measure of induced pain, was not statistically different between KO and WT mice (Price et al., 2001). They also demonstrated enhanced sensitivity of rapidly adapting Aβ mechanoreceptors, reduced firing frequency and increased activation threshold of Aδ fibers in KO mice. Polymodal C-fiber nociceptors examined in these experiments displayed impaired acid sensitivity. Thus, disruption of ASIC3 genes failed to abolish any of the sensory modalities completely, while the sensory transduction was definitely altered.

On the isolated ASIC3−/− DRG neurons it was demonstrated that the effect of FMRFa on the amplitude and desensitization of the responses to acid was significantly reduced, but not abolished completely; it was suggested that ASIC1 was responsible for the effect of RFa peptide on ASIC3 −/− neurons (Xie et al., 2003). In case of NPSF algogenic action is mediated by the modulation of ASIC3 one could expect that the disruption of ASIC3 gene would alter the pain-related behavior induced by NPSF. However, the loss of ASIC3 gene did not result in any changes. According to the data of Chen et al. (2002), there is no compensatory up-regulation of other ASICs genes in KO mice. Therefore the algogenic action of the RFa peptides on the peripheral nerve endings cannot be interpreted in the term of their action on the ASIC3. It should be noted that FMRFa and NPFF slow down the desensitization kinetics of homomeric ASIC1 as well (Askwith et al., 2000). Correspondingly our result cannot entirely rule out possible involvement of other representatives of ASIC family in the algogenic effect of RFa-peptides. Therefore it is possible that ASIC3 play only minor role in the excitatory action of peripheral RFa peptides as well as in the acid induced pain. It can be suggested that ASIC1 could be responsible for the effect of NPSF in the ASIC3 knockout animals. However, extensive phenomenology indicating at the antiopioid aspect of this peptide may be of high relevance (Gayton, 1982; Gouarderes et al., 1996). Although RFa peptides do not bind to μ- or δ-opioid receptor (Allard et al., 1989; Payza and Yang, 1993), naloxone-dependent liberation of RFa peptides from the brain tissue has been reported in response to the administration of opioid (Devillers et al., 1995).

Thus, NPSF increases the number of algogenic RFa-related peptides. Its action has no apparent link with the modulatory effect on the ASIC3, which is specific for the sensory neurons. Thus, the molecular targets for the pain-producing peripheral action of RFa peptides remain to be disclosed.

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