DOI 10.1007/s11055-017-0402-6
Neuroscience and Behavioral Physiology, Vol. 47, No. 3, March, 2017

Effects of Neuropeptide Y on Neuron Spike Activity in the Rat Suprachiasmatic Nucleus in Vitro

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Translated from Rossiiskii Fiziologicheskii Zhurnal imeni I. M. Sechenova, Vol. 101, No. 11, pp. 1257–1269, November, 2015. Original article submitted April 8, 2015. Revised version received September 2, 2015.

Experiments on sagittal hippocampus slices from male Wistar rats addressed the effects of 10 nM neuropeptide Y on the level of electrical activity in neurons in the suprachiasmatic nucleus and parameters of spike-based information encoding. Application of neuropeptide Y led to a decrease in the action potential generation frequency in 35 of the 81 neurons recorded; eight cells showed increases in this parameter, and the remaining 38 neurons showed no change in the level of spike activity. The decrease in the spike generation frequency in suprachiasmatic nucleus neurons was accompanied by increases in the entropy of the distribution of interspike intervals and the mutual information between adjacent interspike intervals, which is evidence for increases in the degree of irregularity in these intervals and the patterning of spike information in response to neuropeptide Y. These results demonstrate the ability of neuropeptide Y to modulate the activity level and affect the spike code in the relatively numerous population of neurons in the circadian oscillator of the suprachiasmatic nucleus.

Keywords: neuropeptide Y, suprachiasmatic nucleus, spike activity, spike code.

The suprachiasmatic nucleus in mammals contains a biological clock generating an endogenous circadian rhythm and regulating numerous circadian physiological, homeostatic, and behavioral rhythms [1, 15]. Afferentation from three sources plays an important role in tuning the phases and period of the circadian rhythm of the suprachiasmatic nucleus: from retinal photoreceptors via the retinohypothalamic pathway, from the median raphe nuclei via the serotoninergic pathway, and from the intergeniculate leaflet of the thalamus via the geniculohypothalamic pathway [9]. Among these afferent pathways, neurophysiologists currently pay particular attention to the geniculohypothalamic pathway. This is due particularly to the fact that the intergeniculate leaflet is rich with connections (mainly bilateral and reciprocal) with other parts of the brain [23]. The number of areas approaches a hundred, and all these regions can theoretically take part in regulating circadian rhythms via the geniculohypothalamic pathway, which is made up of fibers mainly containing neuropeptide Y [23, 28].

A number of studies have yielded data evidencing the direct involvement of neuropeptide Y in modulating the functioning of the circadian oscillator. Thus, lesioning of the origin of the geniculohypothalamic pathway, eliminating the release of neuropeptide Y in the suprachiasmatic nucleus, impairs non-photic tuning of circadian rhythms in locomotor activity [17, 18, 35] and photic tuning of circadian rhythms in body temperature and behavior [8, 24], while electrical stimulation of the geniculohypothalamic pathway leads to phase shifts in the locomotor activity rhythm [27]. There are good grounds for believing that the circadian effects of neuropeptide Y are due primarily to its direct actions on the activity of neurons in the suprachiasmatic nucleus. Previous in vitro experiments demonstrated the ability of neuropeptide Y to evoke decreases in the level of electrical activity of neurons in the suprachiasmatic nucleus in hamsters and mice [3, 22]. However, these studies addressed the effects of neuropeptide Y on a single measure

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of cell activity – the mean action potential generation frequency. Our previous studies showed that circadian oscillator neurons display circadian rhythms in other measures of the spike code, particularly the entropy of the distribution of interspike intervals, which is a measure of the inhomogeneity of their durations, and the mutual information between adjacent interspike intervals, which reflects the degree of patterning of spike information [6]. Our subsequent in vitro studies demonstrated the ability of chemical modulators (especially melatonin and leptin) to influence measures of the spike code of suprachiasmatic nucleus cells [13, 14].

The present study, performed in vitro using living slices of rat hypothalamus, addressed the effects of neuropeptide Y on the level of spike activity and measures of the spike code of suprachiasmatic nucleus neurons.

Methods

Experiments were performed on male Wistar rats weighing 70–160 g. The experimental protocol was agreed with the Samara State University Biological Ethics Committee. Rats were anesthetized with urethane (1.2 g/kg i.p.) and decapitated. The brain was extracted from the skull cavity, cooled in artificial cerebrospinal fluid at 1–3°C, and then sectioned with a vibratome (Vibroslice NVSL, WIP, USA) to produce sagittal slices of hypothalamus of thickness 300 μm including the suprachiasmatic nucleus. Slices were incubated for at least 21 h in oxygen-saturated artificial cerebrospinal fluid at 37°C to the point at which recording began. Artificial cerebrospinal fluid contained 124 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 mM NaH_2PO_4, and 30 mM glucose. For recording, slices were placed in a Plexiglas chamber mounted on an antivibration platform (Vibraplane, USA) to increase recording stability. Slices were perfused at a constant rate of 1.5 ml/min with a peristaltic pump (Minipuls 3, Gilson, France). Recordings were made at 27–30°C.

The spike activity of neurons in the suprachiasmatic nucleus was recorded using glass microelectrodes with tip diameters of about 1 μm filled with the artificial cerebrospinal fluid used for perfusion of the slices. Microelectrode signals were amplified (2400 A, Dagan, USA), 50-Hz noise was removed (Hum Bug, Quest Scientific, Canada), and signals were digitized (Micro 1401, CED, UK) and recorded on a personal computer. Signals were visualized, stored, and subjected to primary processing in Spike-2 (CED, UK).

Recordings were made during subjective daytime (CT 04:00 to 12:00), as the effects of neuropeptide Y on the level of "population" activity in the suprachiasmatic nucleus does not change during the subjective day [10]. After detection, spike activity was monitored in the initial state for at least 10 min to ensure stable action potential generation frequency. If there was no marked trend to variation in this this parameter, perfusion was changed to solution of the same composition but containing 10 nm neuropeptide Y for 10 min, followed by return to the initial solution to wash slices to remove peptide. Possible desensitization was excluded by recording just one application of neuropeptide Y to each slice. The duration of washing was 15 min.

The first stage of data processing consisted of careful separation of all the spikes recorded from noise and artifacts. Further, along with calculation of the "traditional" measure of neuron electrical activity, i.e., mean spike generation frequency, two additional parameters were calculated to characterize spike-based information coding: the entropy of the distribution of interspike intervals and the mutual information between adjacent interspike intervals, reflecting the patterning of spike information [4–6, 13, 14]. The entropy of the distribution of interspike intervals was calculated using the Shannon formula [30]:

$$S(X) = -\sum_{i=1}^{N_x} P(x_i)\log_2 P(x_i),$$

where $S(X)$ is the entropy of the probability histogram of the distribution of the durations of the set of interspike intervals analyzed, $P(x_i)$; N_x is the number of bins in the histogram $P(x_i)$. Mutual information between adjacent interspike intervals was calculated as follows. In pairs of adjacent intervals, the set of logarithms of the preceding intervals was designed X and that of subsequent intervals as Y. Mutual information between adjacent interspike intervals was defined as the difference between the relative entropy of the duration of interspike intervals in the order in which they were recorded, and the mean relative entropy of the same data taken in random order after a randomization procedure using the Monte Carlo method [4]:

$$I(X;Y) = D(X, Y \parallel XY) - \bar{D}(X, R_iY \parallel XY),$$

where $I(X; Y)$ is the mutual information between adjacent interspike intervals, $D(X; Y \parallel XY)$ is the relative entropy of the duration of interspike intervals in the order in which they were recorded in the experiment, R_iY is the randomized set of intervals Y, and $\bar{D}(X; R_iY \parallel XY)$ is the mean relative entropy of the duration of interspike intervals after randomization.

The effects of neuropeptide Y were identified by comparing spike activity parameters during two 5-min time periods: in the baseline state (immediately before peptide application) and at the end of the application period. The only neurons regarded as responding to neuropeptide Y were those in which the spike general frequency changed in response to this substance by at least 20% of the baseline level [7]. This was followed by analysis of the study parameters during the final 5-min period of washing the slice and determination of the extent of recovery of the baseline activity of the neuron.

The experimental data were analyzed statistically. Parameter values obtained during experiments were compared with baseline using the paired t test or the Wilcoxon rank test (when data distributions were not normal). Distri-

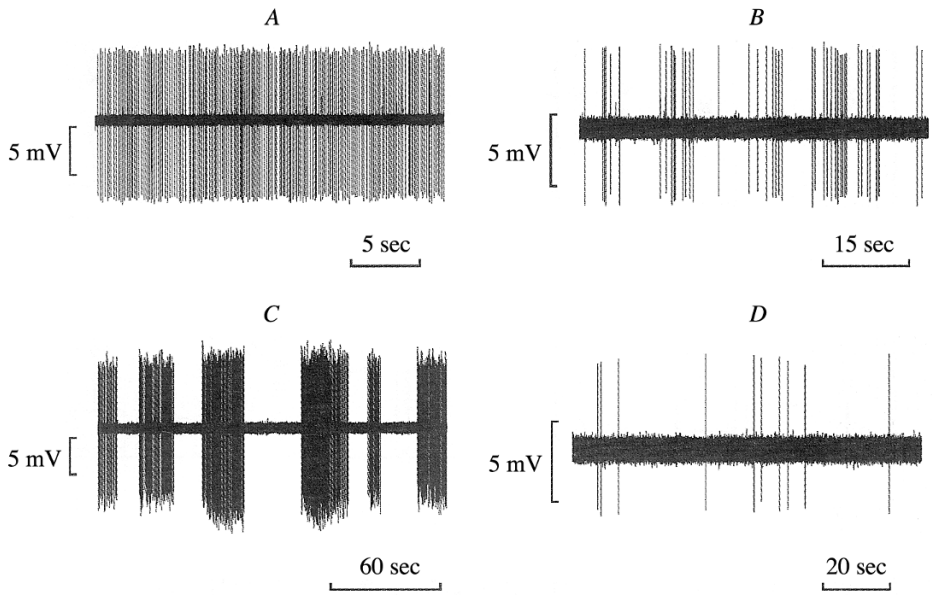

Fig. 1. Types of in vitro suprachiasmatic nucleus neuron spike activity. *A*) Regular activity; *B*) irregular activity; *C*) volley activity; *D*) low activity. Amplitude calibration is shown to left of each plot (5 mV); time calibration is shown beneath each plot (sec).

bution normality was determined using the Shapiro–Wilk test and the equality of variances using the Levene test. Statistical data on measures of neuron spike activity in the baseline state are presented as arithmetic mean ± standard error of the mean. Changes in study parameters were regarded as statistically significant at $p < 0.05$.

Results

Activity was recorded from a total of 81 neurons in the suprachiasmatic nucleus. The overall action potential generation frequency for the whole set of neurons was 2.62 ± 0.29 sec^{-1}. The entropy of distribution of interspike intervals for these neurons was 6.62 ± 0.11 bits and the mutual information between adjacent interspike intervals was 0.056 ± 0.013 bits. Analysis of the characteristics of spike activity identified four different types of neuron and typical examples of these are presented in Fig. 1.

Regular activity was seen in 24 of the 81 neurons recorded (29.6%). The most typical features of this activity were the higher (as compared with other neuron types) action potential generation frequency (4.98 ± 0.55 sec^{-1}) combined with a stable interspike interval duration and the complete absence of pauses between neighboring spikes (Fig. 1, *A*). The high regularity of spike generation by neurons of this type was apparent, particularly, in the lowest (of all neuron types) entropy of distribution of interspike intervals, which was 5.44 ± 0.09 bits. Mutual information between adjacent interspike intervals in cells of this type was 0.033 ± 0.007 bits, which points to a relatively low level of patterning of information in the neural code.

The most frequent type of suprachiasmatic nucleus neuron activity, recorded in 36 of 81 cells (44.4%), was the irregular type of spike activity. This was characterized by irregularity of interspike intervals, short periods of high-frequency activity, and occasional brief pauses, generally lasting no more than 10 sec (Fig. 1, *B*). Neurons of this type displayed moderately high spike generation frequencies (2.30 ± 0.33 sec^{-1}) and interspike interval distribution entropy (6.95 ± 0.09 bits). Mutual information between adjacent interspike intervals for neurons of this type was 0.065 ± 0.022 bits, which is evidence of a moderate degree of information patterning in the spike code of these neurons.

Fifteen of the 81 cells recorded (18.5%) were classified as neurons with low activity (Fig. 1, *D*). The most characteristic feature of the these cells was the low action potential generation frequency, which was no greater than 0.35 sec^{-1} and averaged 0.18 ± 0.03 sec^{-1} for the whole group. Another typical feature of these neurons, as compared with all others, was the highest entropy of the distribution of interspike intervals (7.70 ± 0.16 bits), as well as the extremely low level of mutual information between adjacent interspike intervals (with a null value in 14 of 15 of these neurons). Thus, the spike code of these cells was characterized by a high level of irregularity in action potential generation and the virtually complete absence of signs of information patterning.

Finally, the rarest cells (six out of 81, 7.4%) were those with volley activity, which were characterized by alternating periods of activity (volleys) and long pauses. Volley duration ranged from a few seconds to several minutes, and the durations of the pauses between volleys generally ranged from 10–15 to 60 sec (Fig. 1, *C*). One of the most characteristic features of the activity of volley neurons was

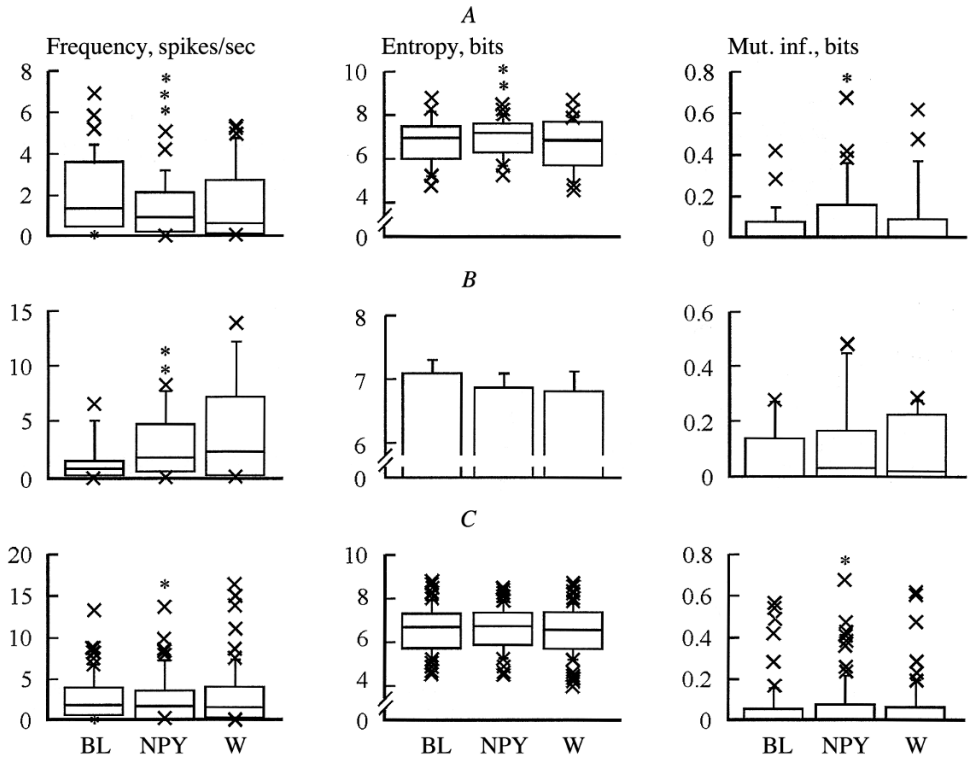

Fig. 2. Effects of 10 nM neuropeptide Y on action potential generation frequency (plots at left), the entropy of the distribution of interspike intervals (central plots), and mutual information between adjacent interspike intervals (plots at right). *A*) Neurons whose activity decreased in response to neuropeptide Y ($n = 35$); *B*) neurons whose activity increased in response to neuropeptide Y ($n = 8$); *C*) the whole set of neurons recorded ($n = 81$). BL – baseline; NPY – exposure to neuropeptide Y; W – after washing with artificial cerebrospinal fluid. Significant differences compared with baseline: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Wilcoxon rank test).

the highest, as compared with all cell types, median mutual information between adjacent interspike intervals (0.161 ± 0.092 bits), indicating a high level of spike information patterning capacity in the neural code. The entropy of the distribution of interspike intervals was also relatively low (6.66 ± 0.38 bits), as was the action potential generation frequency (1.23 ± 0.23 sec^{-1}), which can be explained by the long pauses between activity volleys and the regularity of spike generation during volleys.

Addition of 10 nM neuropeptide Y to the perfusion solution decreased the mean spike generation frequency in 35 of the 81 suprachiasmatic nucleus neurons recorded (43.2%) (Fig. 2, *A*). This group of cells included 16 neurons with irregular-type activity, seven with regular activity, seven with low activity, and five with volley activity. The median spike generation frequency of neurons of this group decreased from 1.33 to 0.95 sec^{-1} ($p < 0.001$, Wilcoxon rank test). Another characteristic feature of the response of these cells was an increase in the entropy of the distribution of interspike intervals, with an increase in the median value from 6.92 to 7.16 bits ($p = 0.005$, Wilcoxon rank test), which evidences an increase in the degree of irregularity of spike generation in the neural code in response to neuropeptide Y. This was accompanied by a simultaneous statistically significant increase in the mutual information between adjacent interspike intervals ($p = 0.020$, Wilcoxon rank test), pointing to an increase in the degree of information patterning in the spike code. Typical examples of the responses to application of neuropeptide Y, consisting of reductions in the level of spike activity in neurons initially with irregular and volley activity, are shown in Figs. 3 and 4.

In eight cases (9.9%), application of 10 nM neuropeptide Y to the perfusion solution led to an increase in the action potential generation frequency by suprachiasmatic nucleus cells (Fig. 2, *B*). These cells were six neurons with irregular activity and two neurons with low activity. The median spike generation frequency of neurons demonstrating this type of activity increased from 0.76 to 1.71 sec^{-1} ($p = 0.008$, Wilcoxon rank test). Neuropeptide Y had no statistically significant effects on the entropy of the distribution of interspike intervals ($p = 0.116$, paired t test) or mutual information between adjacent interspike intervals ($p = 0.250$, Wilcoxon rank test) in these neurons. An example of the response to application of neuropeptide Y, consisting of an increase in spike generation frequency in neurons with initially irregular activity is shown in Fig. 5.

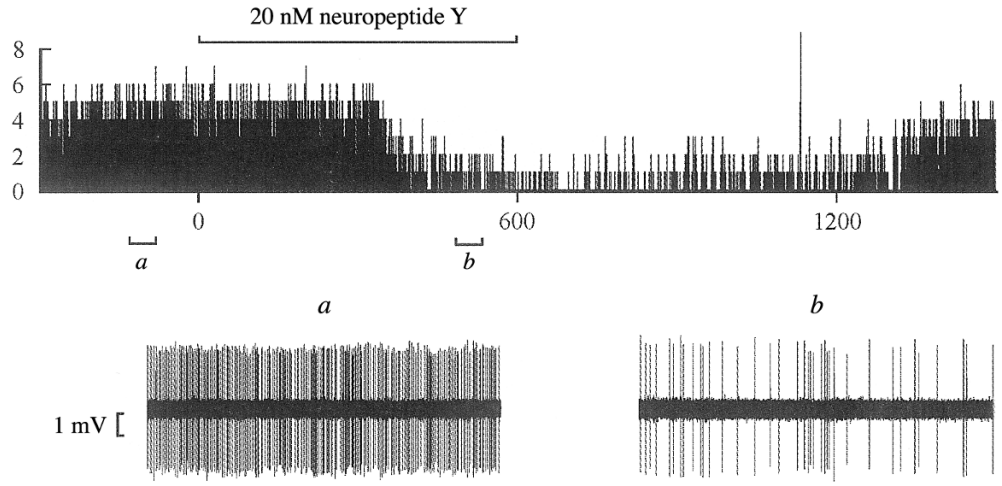

Fig. 3. Examples of the responses of a suprachiasmatic nucleus neuron with initial irregular activity in response to application of 10 nM neuropeptide Y. The upper part shows a histogram showing changes in the level of spike activity of the neuron during the experiment. The neuropeptide Y application period is showed by the horizontal bar above the histogram. The abscissa shows time, sec (application started at the point marked 0); the ordinate shows spike generation frequency (sec^{-1}). The horizontal bars beneath the histogram (*a* and *b*) show two 50-sec intervals of the spike activity trace presented below: *a*) immediately before exposure; *b*) at the end of application of neuropeptide Y.

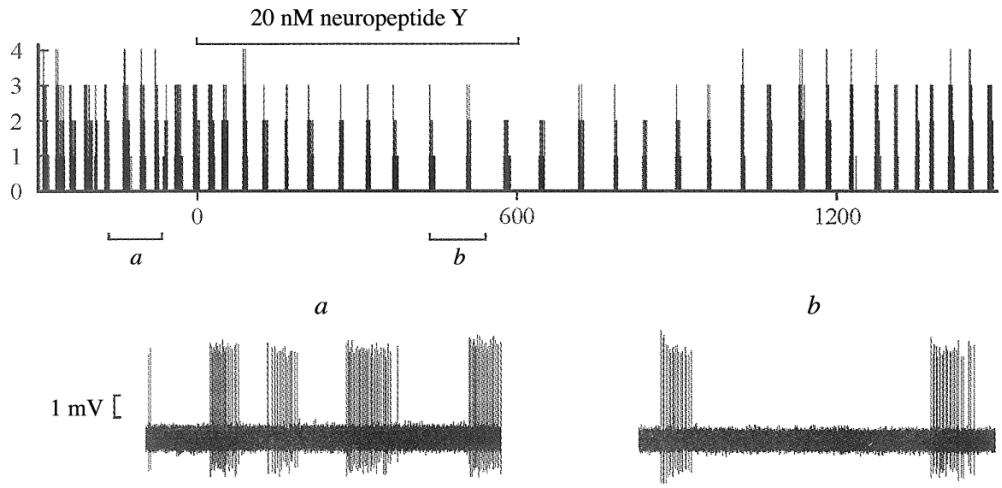

Fig. 4. Example of the responses of a suprachiasmatic nucleus neuron with initial volley activity to application of 10 nM neuropeptide Y. The horizontal bars beneath the histogram (*a* and *b*) correspond to the 100-sec spike activity traces shown below: *a*) immediately before exposure; *b*) at the end of application of neuropeptide Y. For further details see caption to Fig. 3.

There were no significant changes in spike activity levels in response to application of 10 nM neuropeptide Y to the remaining 38 neurons. This group of cells included 17 neurons with regular activity, 14 neurons with irregular activity, six neurons with low activity, and one volley-active neuron. There were no statistically significantly changes in action potential generation in these cells (median of 2.56 sec^{-1} in the initial state and 2.59 sec^{-1} on exposure to neuropeptide Y, $p = 0.331$, Wilcoxon rank test). This group of neurons also showed no changes in the entropy of the distribution of interspike intervals, which was 6.37 ± 0.16 bits before exposure and 6.34 ± 0.15 bits after exposure to neuropeptide Y ($p = 0.457$, paired t test). There was also no change in the mutual information between adjacent interspike intervals ($p = 0.922$, Wilcoxon rank test).

Analysis of reactions to neuropeptide Y in 81 of the suprachiasmatic nucleus neurons recorded as a single group (Fig. 2, *C*) showed that the peptide induced a weak but statistically significant decrease in spike generation frequency. The medial value decreased from 1.85 to 1.72 sec^{-1} ($p = 0.011$, Wilcoxon rank test). This was accompanied by a simultaneous increase in mutual information between adjacent inter-

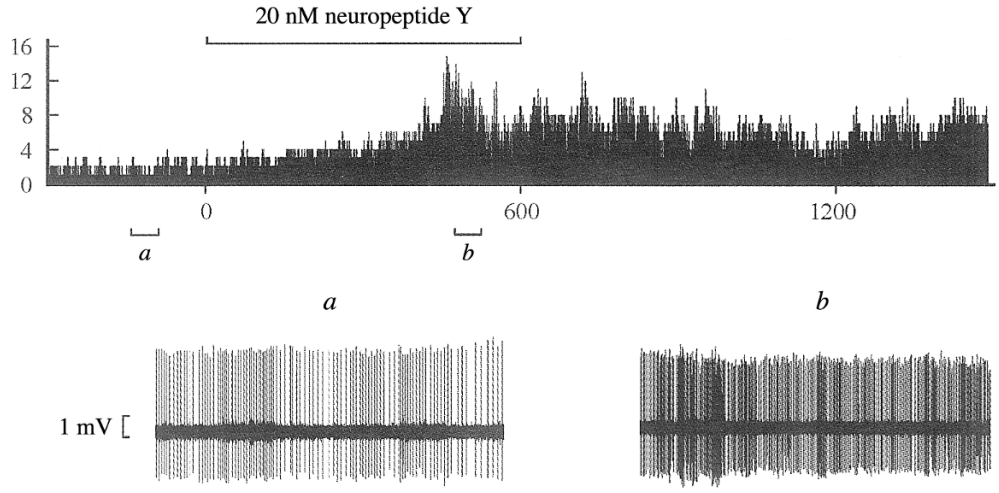

Fig. 5. Example of the response of a suprachiasmatic nucleus neuron with initial regular activity to application of 10 nM neuropeptide Y. For further details see caption to Fig. 3.

spike intervals ($p = 0.037$, Wilcoxon rank test), though there were changes in the entropy of the distribution of interspike intervals ($p = 0.237$, Wilcoxon rank test).

Overall data on the quantitative distribution of reactions to neuropeptide Y, consisting of decreases or increases in activity, and on the numbers of cases without reactions among suprachiasmatic nucleus neurons with different types of baseline activity, are shown in Table 1.

The reversibility of spike activity reactions to neuropeptide Y was analyzed by comparing measurement values at the end of the 15-min washout period with the baseline activity. There were no statistically significant differences in spike activity in baseline conditions and after washing (Fig. 2), evidencing complete or partial restoration of baseline activity. This is shown by the reactions of the individual neurons shown in Figs. 3–5, which display a tendency to recover initial action potential generation frequencies.

Discussion

This study addressed the effects of neuropeptide Y on the level of spike activity and measures of the spike-based information encoding by suprachiasmatic nucleus neurons in vitro. Four types of neuron were identified, with different spike activity characteristics: neurons with irregular, regular, low, and volley activity. A series of previous in vitro studies using extracellular and intracellular recording of the activity of individual suprachiasmatic nucleus neurons also yielded data on the existence of different types of spike activity (regular, irregular, volley) and the existence of a significant proportion of "silent" cells, with low activity levels or no activity, in this nucleus [7, 16, 19, 20, 26, 32]. The approach used here to analyze spike activity, which involved not only the action potential generation frequency, but also measures of spike-based information encoding (entropy of distribution of interspike intervals and mutual information between adjacent interspike intervals), was valuable in relation to quantitative analysis of the neural code and in identifying types of cell activity.

Application of neuropeptide Y was found to affect the level of spike activity in 53.1% of suprachiasmatic nucleus neurons, no significant changes being seen in the other cells. This result may reflect the proportion of suprachiasmatic nucleus neurons involved in processing specific neuropeptide Y receptors. The dominant reaction, seen in 43.2% of cases, consisted of a decrease in the action potential generation frequency. Only 9.9% of cases showed increases in the baseline action potential generation frequency in response to this peptide. This result is consistent with data reported by other authors obtained in experiments on mouse and hamster brain slices, identifying mainly suppressive influences of neuropeptide Y on spike activity levels in suprachiasmatic nucleus neurons [3, 22]. Overall, the differences in the directions of the spike activity reactions of individual neurons seen here may, in particular, be explained by activation of different types of specific Y receptors in suprachiasmatic nucleus neuron membranes, and also by the involvement of different intracellular messengers in the reaction.

As information is transmitted by CNS neurons in terms of the corresponding pattern of spike activity, the present study of the effects of neuropeptide Y is the first using measures of spike-based encoding of information to provide a more complete characterization of cell activity. Decreases in activity levels in suprachiasmatic nucleus neurons arising in response to neuropeptide Y were found to occur in conditions of increases in the entropy of the distribution of interspike intervals and mutual information between adjacent interspike intervals. This type of reaction is evidence that study peptide increased the irregularity of action potential generation in these neurons and the degree of patterning of spike information in the neuron code. At the same time, neurons whose activity levels increased in response

TABLE 1. Numbers of Reactions in Different Directions to Application of Neuropeptide Y among Suprachiasmatic Nucleus Neurons with Different Initial Types of Activity

Type of activity	Decreased activity	Increased activity	No response
Irregular (n = 36)	16*	6	14
Regular (n = 24)	7*	–	17
Low (n = 15)	7	2	6
Volley (n = 6)	5*	–	1
Total (n = 81)	35***	8	38

Notes. Statistically significant differences between the frequencies of responses to neuropeptide Y consisting of decreases and increases in mean action potential generation frequency in groups of neurons with different types of initial spike activity: *$p < 0.05$, ***$p < 0.001$ (z test).

to neuropeptide Y showed no statistically significant changes in measures of spike-based information encoding. These results may point to fundamental differences between the characteristics of neuron populations demonstrating changes in activity levels in opposite directions in response to neuropeptide Y. This suggestion is also supported by differences in the types of spike activity in cells responding to neuropeptide Y with decreases and increases in activity. In fact, neuron populations whose activity levels increased in response to peptide completely lacked cells with regular and volley-type activity, while neurons with responses in the opposite direction included cells with all four types of baseline activity.

The possible mechanisms of action of neuropeptide Y at the level of suprachiasmatic nucleus neurons continue to be studied. There are good grounds for suggesting that the leading role in mediating the effects of this substance is played by specific neuropeptide Y receptors. At least three types of these receptors are expressed in the suprachiasmatic nucleus: Y1, Y2, and Y5 receptors [12, 21, 34]. Data have been reported showing that the inhibitory influence of neuropeptide Y may be based on activation of a particular type of potassium channel in the membranes of suprachiasmatic nucleus neurons [11]. These channels may, in particular, be G protein-coupled inwardly-rectifying potassium channels (GIRK). Previous studies have demonstrated the ability of neuropeptide Y to activate the inwardly-rectifying potassium current in different parts of the brain in mammals, including those located close to the suprachiasmatic arcuate nucleus of the hypothalamus [2, 25, 31]. Although this current in suprachiasmatic nucleus neurons remains to be identified, an answer to this issue may be an important step in understanding the mechanisms of action of neuropeptide Y and non-photic tuning of the circadian oscillator in general. Another electrophysiological study on suprachiasmatic nucleus slices and cell cultures [33] showed that 8-min application of neuropeptide Y induces prolonged decreases in EPSC amplitude, membrane hyperpolarization, and decreases in the intracellular calcium ion concentration for long periods (more than 1.5 h). The authors obtained data providing grounds for suggesting that the long-term effects of neuropeptide Y can be explained in terms of its modulatory influence on the presynaptic membranes of glutamatergic axons. At the same time, the effects of this peptide at the level of the postsynaptic membrane, mediated via Y1 and Y2 receptors, are short-lived and transient. Recent studies have demonstrated that this peptide elicits long-lasting decreases in the excitation of the cell population of the suprachiasmatic nucleus expressing the *Per-1* gene [3]. Despite evidence pointing to a link between the long-term effects of neuropeptide Y and the phases of the circadian cycle, relatively quick and marked suppression of spike activity can be seen in any phase of the cycle [10]. It remains possible that the inhibitory effect of neuropeptide Y on the level of spike activity and the ability of this substance to evoke an anticipatory phase shift in the circadian rhythm are mediated via different types of specific receptors. In fact, activation of Y5 receptors led to suppression of activity and had no effect on the phase of the rhythm, while activation of Y2 receptors evoked phase anticipation of the spike activity rhythm [10]. These effects of neuropeptide Y may to some extent be linked with activation of the enzyme protein kinase C, as previous in vitro studies [29] demonstrated a significant increase in the activity of this enzyme in the suprachiasmatic nucleus of hamsters during the 15-min time period after application of peptide.

Overall, the results of these in vitro studies show that neuropeptide Y can, when applied directly, modulate the level of activity and influence the spike code of the relatively large population of neurons in the circadian oscillator of the suprachiasmatic nucleus. Given that the main source of neuropeptide Y in the suprachiasmatic nucleus consists of the terminals of the fibers of the geniculohypothalamic pathway, these data identify the features of the spike activity reactions of circadian oscillator neurons taking place as part of the mechanisms of non-photic tuning involving this afferent pathway and endogenous neuropeptide Y.

This study was supported by the Russian Foundation for Basic Research (Grant Nos. 10-04-00653-a and 13-04-97000-r_Povolzhie_a).

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