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Physiological evidence for two classes of mitral cells in the rat olfactory bulb

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Abstract

The spontaneous activity of mitral cells was recorded in vivo from the main olfactory bulb of freely breathing anesthetized rats. Single units recorded extracellularly from the mitral cell body layer were further identified as mitral cells by antidromic activation of the lateral olfactory tract and the posterior piriform cortex. Hierarchical cluster analysis of their spontaneous activity showed that at least two classes of mitral cells could be distinguished. A post-hoc multivariate analysis of variance indicated significant differences between the two groups based on mean rate, latency, and the coefficient of variation in interspike interval. Univariate tests showed that the groups differed in mean rate, but not in latency, or in the coefficient of variation in interspike interval. Autocorrelation analysis showed that the high frequency group tended to fire in bursts. Functional implications of these putative subclasses of mitral cells are discussed.

1. Introduction

Classic experiments in the visual system (Weisel & Hubel 1963; Hubel & Weisel, 1970) demonstrated the importance of sensory experience during critical periods in maintaining cortical circuitry. Subsequently, Katz & Shatz (1996) highlighted the importance of spontaneous activity in the development of thalamic synaptic connections. More recently, Yamada et al. (2010) demonstrated that both pre and postsynaptic neural firing markedly affected axon branching during development in coculture preparations of thalamus and cortex.

In the olfactory system recent evidence suggests that both presynaptic (Yu et al., 2004; Rospars et al., 1994) and postsynaptic (Biju et al., 2008) activity may be required for the establishment and/or maintenance of a normal olfactory sensory map. Tremendous progress has been made in understanding how olfactory receptor neurons (ORNs) converge onto and convey information to the main olfactory bulb (MOB). An important and as yet incompletely answered question is how mitral cells, one class of output neurons from the MOB, form and maintain appropriate neural circuits in the bulb and olfactory cortex to encode odor information.

In the olfactory system, mitral and tufted cells provide parallel pathways from the MOB, and in other sensory systems such parallel pathways carry different aspects of sensory information. Three subclasses of tufted cells based on the position of their somata, and two

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subtypes of mitral cells based on the location of secondary dendrites have been described (Orona et al, 1984; Macrides et al., 1985). Mitral cells, but not tufted cells, are the major source of afferent input to the piriform cortex, the largest olfactory cortical area. Thus, physiological subclasses of mitral cells capable of coding different aspects of odor quality are also expected.

There have been many studies of mitral cells, including excellent single unit recordings in mammalian preparations. However, only a few studies have measured and reported the mean rate for the spontaneous activity; many more have examined a stimulus-evoked response and the spontaneous activity was estimated from a short pre-stimulus period. These previous estimates of spontaneous activity show a high degree of variability ranging from 0 to 42 spikes/sec (Green et al., 1962; Phillips et al., 1963; Mair, 1982; Shepherd, 1963; Yamamoto et al., 1963; Getchell & Shepherd, 1975; Chaput & Holley, 1980; Mori & Takagi, 1978; Harrison & Scott, 1986; Meredith, 1986; Chaput & Lankheet, 1987; Imamura et al., 1992; Yu et al., 1993; Ogawa, 1998; Nagayama et al., 2004; Rinberg et al., 2006). These studies are subject to a variety of methodological problems, including: uncertainty of the identity of the cell as a mitral cell; effects of anesthesia; bias in selecting/isolating cells; and short lengths of recording. As a result of these problems, the spontaneous activity of mitral cells is not really known.

In this paper we test the hypothesis that there may be subclasses of mitral cells based on the rate and temporal patterns of their spontaneous activity. Such differences could play a role in the development and/or maintenance of functionally important synaptic connections in the MOB and/or piriform cortex analogous to suggestion that the formation of functional sublaminae in the lateral geniculate nucleus for the ON and OFF subclasses ganglion cells could be driven by differences in the spontaneous firing patterns of those cells in the vertebrate retina (Katz & Shatz, 1996).

2. Results

Mitral cells identified by antidromic activation and histological confirmation (N = 29) showed considerable variability (Fig.1). Spontaneous activity varied across units from 4.6 to 35.0 spikes/s, latency from 2.0 to 11.4 ms, and the coefficient of variation (CV) in interspike interval from 0.87 to 7.11 (Table 1). The mean spontaneous activity for all mitral cell recordings was 17.38 ± 8.3 spikes/s.

Hierarchical cluster analysis showed that at least two classes of mitral cells could be distinguished. Eight cells were placed in one group (Group A, Table 1) and 21 in the other (Group B). *Post-hoc* MANOVA indicated significant differences between the two groups based on mean rate, latency, and CV in interspike interval (Wilks' $\lambda = 0.1741$, $F_{3,25} = 40.47$, $P < 0.001$). Examination of *Post hoc* univariate tests showed that the groups differed in mean rate ($F_{1,27} = 106.91$, $P < 0.01$), but not in latency ($F_{1,27} = 1.79$, $P = 0.16$) or in the CV of the interspike interval ($F_{1,27} = 0.01$, $P = 0.92$). There was greater variation in the latency for the fast group of mitral cells (Group A) than for the slow group, (Group B), ($F_{1,27} = 5.83$, $P = 0.02$). The higher frequency group (Group A) had a mean of 28.94 ± 4.7 spikes/s compared to 12.87 ± 3.34 spikes/s for the lower frequency group (Group B).

The groups also showed some difference in their firing pattern based on their temporal autocorrelation (Fig. 3). Both groups showed a tendency to discharge with a relatively short lag following any given spike. For cells in Group A, there was a strong autocorrelation ($\Phi = 0.10$) at lag 2 (2-4ms) decaying to ($\sim\Phi = 0.02$) after 20 ms. For cells in Group B, the strongest autocorrelation was at lag 7, 12-14 ms ($\Phi = 0.03$), but varied little between 2 and 20 ms following a discharge. The cells in Group A tended to discharge in short bursts,

several at a time, whereas those in Group B tended to discharge with less regularity and fewer subsequent spikes following a given action potential (see also Fig. 1, insets).

3. Discussion

All mitral cells in this study were identified by antidromic activation from the posterior piriform cortex and by histological confirmation of a recording site in the mitral cell body layer for most units. Antidromic activation of axons in the lateral olfactory tract (LOT) can identify neurons that project from the MOB, but in many previous studies data from mitral and tufted cells were combined, and the cells were referred to as 'mitral/tufted' or 'M/T' cells (e.g., Buonviso et al., 1992; Imamura et al., 1992; Yokoi et al., 1995). In other prior studies (e.g., Mair, 1982; Chaput & Holley, 1980), cells were identified by depth in the MOB, but without antidromic activation. Such cells could be mitral cells, granule cells, or deep tufted cells. Antidromic activation in our study would also have allowed us to detect cells with no or very low spontaneous activity, though none were found.

The mean rate of spontaneous activity in this study of 17.38 ± 8.3 spikes/s is similar to the mean rate of 14.7 ± 6.2 spikes/s reported by Griff et al. (2008) where the slightly lower rate could be due to a different strain of rat (Wistar versus Sprague Dawley), slightly different anesthetic (equithesin which contains chloral hydrate versus only chloral hydrate) or a deeper plane of anesthesia. The lower value reported by Nagayama et al. (2004) of 7.4 ± 6.6 could reflect differences in the strain (Wistar), anesthetic (urethane), depth of anesthesia, and/or selection of cells from the dorsomedial MOB.

Our cluster analysis identified 2 groups of mitral cells, one with mean rate of 28.94 and the other with a mean rate of 12.87 spikes/s; we refer to cells in the higher rate as Group A and the lower as Group B to distinguish them from the subclasses of mitral cells based on morphological data that were identified by Orona et al. (1984), called Group I and II. Group I mitral cells had the deepest and longest secondary dendrites and lacked axon collaterals; Group II mitral cell dendrites arborized in an intermediate zone of the external plexiform layer and axon collaterals were present. Wellis et al. (1989) presented intracellular electrophysiological responses and anatomical data for a mitral cell that appears to be a Group I mitral cell. The spontaneous activity, though not explicitly reported in the Wellis paper, is about 23 spikes/s, and the temporal pattern of action potentials appears to burst; this fits the rate and general pattern of Group A mitral cells in the current study. If this recording is typical of the Group I mitral cells, one could hypothesize that the Group A subclass of mitral cells based on physiological measurements in the present study coincides with the Group I cells of Orona et al. (1984) based on histology.

The faster Group A mitral cells more often fire action potentials in short bursts as shown by interspike interval histograms with bursts separated by 10-30 ms inter-burst intervals (Fig. 1A). Several ranges of oscillations at the population level have been identified in odor-evoked local field potentials in the MOB associated with different behaviors (reviewed in Kay et al., 2009). For example, theta oscillations (1-12 Hz) are associated with changes in the respiratory cycle and exploratory sniffing. Beta oscillations (15-30 Hz) induced by odors are modulated by and potentially involved with associative conditioning (Gervais et al., 2007). Though huge gaps exist between understanding the initial detection of an odor by an animal and subsequent modulation of respiration and/or learning, it is intriguing to speculate that the spontaneous activity of a subclass of mitral cells may play a role.

The source of spontaneous activity in mitral cells is not well understood, and it could reflect synaptic and/or intrinsic membrane properties that trigger or generate this activity. In frogs, partial surgical deafferentation of mitral cells significantly increased spontaneous activity

(Rospars et al., 1994), suggesting a tonic inhibition of mitral cells in frog via inhibitory interneuron circuits that are tonically activated by spontaneous activity of olfactory receptor neurons. In rats, though olfactory receptor neurons display spontaneous activity (e.g., Duchamps-Viret et al., 2005), partial chemical deafferentation using lidocaine did not alter mitral cell spontaneous activity (Griff, unpublished). In both rat and frog, GABA antagonists affect spontaneous activity indicating that local inhibitory circuits and/or inhibitory centrifugal inputs to the MOB modulate mitral cell activity (Duchamp-Viret & Duchamp, 1993; Duchamp-Viret et al., 1993; Yu et al., 1993).

As described briefly in the Introduction, sensory experience and spontaneous activity plays a role in the establishment and maintenance of axonal branching and synaptic connections. Thus, the 2 subclasses of mitral cells suggested by this study could establish different circuits in the olfactory bulb. Such circuits can be analyzed in part by measuring how pharmacological agents and/or genetic manipulations that alter particular synapses or mechanisms affect spontaneous activity. Extracting information about differences in mitral cell circuits from odor-evoked responses is more difficult since each mitral cell can respond to many odorants and the response to each odorant can vary. In the frog, application of GABA_A antagonists directed at glomerular or granule cell circuits increased spontaneous activity and altered the temporal pattern in most mitral cells, but decreased or suppressed activity in about 20% of the cells (Duchamp-Viret et al., 1993; Duchamp-Viret & Duchamp, 1993). A similar result was found in the rat where bicuculline increased spontaneous activity for most mitral cells, but decreased it in 20% of the units (Yu et al., 1993). It is intriguing to consider that this smaller group of mitral cells represents a subtype with higher spontaneous activity. Though only representative examples are presented in the Yu et al. paper, the unit showing a bicuculline-induced decrease in activity had the highest mean rate of the units shown.

More recently, bulbar circuits have been examined in a slice preparation with particular attention to synaptic interactions in the glomerular layer. One class of juxtaglomerular cells, external tufted cells, is spontaneously active (Hayer et al., 2004) and they can excite GABAergic periglomerular cells which in turn produce tonic and phasic inhibition of mitral cells (Shao et al., 2009). Such tonic inhibition of mitral cells would affect spontaneous activity (Pirez & Wachowiak, 2008). Thus, the different subclasses of mitral cells could reflect, at least in part, differential inhibition from juxtaglomerular circuits based on differences in the number or strength of synapses. Additional experiments are needed to determine the physiological and anatomical characteristics of mitral cell subclasses.

In other sensory systems, adult cells with different spontaneous activity have different projections to the sensory cortex and/or code different aspects of the sensory stimulus. In the retina, different mechanisms generate maintained activity in ON and OFF ganglion cells (Margolis & Detwiler, 2007). Spontaneous retinal waves, and in particular bursts of action potentials in retinal ganglion cells may stabilize retinotopic projections to the lateral geniculate nucleus during development and possibly, thereafter (Katz and Shatz, 1996; Butts and Rokhsar, 2001). Spontaneous activity also plays a role in organizing topographic projection in the auditory system (e.g., Kandler et al., 2009). In the auditory nerve there are three functional groups of neurons based on spontaneous activity for each region representing a particular characteristic auditory frequency. Cells with high spontaneous activity are more sensitive to tonal stimulation and have larger diameter axons and higher conduction velocities (Lieberman and Oliver, 1984).

The function(s) of the subclasses of mitral cells is not yet known. In the present study, conduction velocity (latency) was not different between the mitral cell groups. Possibly the temporal pattern (bursting) or anatomical differences such as the extent of lateral dendrites

or specific interactions with inhibitory interneurons in the glomerular or external plexiform layers are more important in coding odor information. Measurements of mitral cell responses to odor stimulation has revealed that some cells are excited while others are inhibited (e.g., Wellis et al, 1989; Nagayama et al., 2004), that the response can depend on odor concentration (e.g., Mair, 1982; Harrison & Scott, 1986, but also Chalansonnet & Chaput, 1998), and that responses in some cells are modulated by respiration (Macrides & Chorover, 1972; Chaput & Holley, 1980; Chaput et al., 1992; Buonviso et al., 2006). This range of response characteristics and their underlying neural circuits could form a more coherent pattern when more is known about the physiological subclasses of mitral cells.

4. Experimental Methods

Anesthesia and surgical procedures

Animals—All procedures adhered to guidelines established by the American Association for the Accreditation of Laboratory Animal Care and were approved by the University of Cincinnati Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering and to reduce the number of animals used. Experiments were performed on adult male Sprague-Dawley rats weighing between 227 and 403 g. Animals were anesthetized with 4% chloral hydrate, ip. The plane of anesthesia was maintained with additional ip chloral hydrate to be deep enough so that a pinch of the paw did not cause a withdrawal reflex, but did desynchronize the EEG (Jiang et al., 1996). All animals breathed freely; they were not tracheotomized.

The cortical EEG was recorded with a bipolar electrode made from 250 μ m stainless steel wires, insulated except for the cut tips with one electrode about 500 μ m longer than the other. A small (3 mm diameter) area of the parietal cortex was exposed by thinning and removing the skull and the dura. The electrode was inserted in the cortex with the shorter electrode about 200 μ m deep and secured to a skull screw with dental acrylic. The EEG was recorded with a differential amplifier (Grass Instruments, model P16), displayed on an oscilloscope (Tektronix, model 5111A) and recorded on a chart (Gould, model 220).

Surgery—Blunt dissection was used to expose the orbit and lateral surface of the temporal bone. The muscles of the orbit and several that attach to the temporal bone were dissected, the temporal process of the zygomatic bone and the zygomatic process of the temporal bone cut, and the eye reflected inferiorly. The bone covering the lateral surface of MOB, the LOT, and the lateral/inferior surface of the posterior piriform cortex (pPC) was thinned and removed. The dura was then removed and the surfaces kept moist with saline.

Isolation and identification of mitral cells

Stimulation and recording electrodes—A recording micropipette was positioned in the mitral cell body layer of the MOB (see below for details), and two bipolar stimulation electrodes also were positioned. One stimulation electrode on the LOT produced antidromic action potentials in the axons of tufted and mitral cells. Stimulation also produced a field potential in the MOB that has a prominent slow negative component, which reverses polarity when the electrode passes through the MCL. A stimulation electrode on the pPC produced antidromic action potentials in mitral cells.

The LOT and pPC stimulation electrodes were constructed from 250 μ m stainless steel wire insulated except where it was bent and exposed to make contact with the tract or cortex. Square-wave pulses of constant current, typically 50 μ s, 200–400 μ A, were applied using Grass stimulators (model S44), stimulation isolation units (SIU5) with 100 k Ω resistors in series to ensure that the currents was minimally affected by variability in tissue resistance

or electrode contact from animal to animal. The stimulator was triggered manually, at a constant rate (usually 0.5 Hz), or externally by a spontaneously generated action potential. Recording pipettes were pulled from thin-walled borosilicate glass capillaries (1.50 mm O.D., 1.12 mm I. D.) on a Brown-Flaming puller (Model P-80) and filled with 2% pontamine blue dissolved in 0.5 M NaCl solution.

Isolating mitral cells—Isolation of mitral cells started by positioning the recording pipette in the MCL based on a measure of the electrode's advancement through the MOB and the reversal depth of the LOT-evoked field potential. Units were isolated and studied only if they could be antidromically activated from the LOT and pPC. The criteria for antidromic activation were constant latency, an abrupt threshold for activation, and collision with a spontaneous spike (when time permitted). Cells that could not be antidromically activated from pPC were not studied further.

Dye deposited from the tip of the recording pipette marked the recording site by passing cycling current (-10 uA, 7 sec on, 3 sec off) through the electrode for 10 min. At the end of the experiment, the animal was perfused with saline followed by 10% formalin via a cardiac puncture to the ascending aorta. Following perfusion with about 300 ml of formalin, the brain was removed and stored first in 10% formalin, and then 10% formalin with 20% sucrose added. The olfactory bulbs were frozen (ethanol and dry ice) and sectioned using a sliding microtome. Sections with a blue spot were mounted, stained with neutral red, dehydrated in alcohol, substituted with xylene and coverslipped. The position of the intense blue spot indicated the recording site, and this position relative to the MCL was recorded. Units recorded outside the MCL were not used in the analysis.

Isolation of single units—Once the recording pipette was positioned near the MCL, units were sought by alternately listening for spontaneous activity and looking for antidromically driven action potentials at each position. The electrode was advanced in 5 - 10 um steps (Kopf hydraulic micropositioner, model 650) through the MCL, repeating the search for units after each movement. Action potentials were amplified and filtered (Dagan, 2400A amplifier), observed on a storage oscilloscope (Tektronix, 5111) and listened to on an audio monitor (Toshiba RT-S801). Once a unit was detected, the electrode was positioned to maximize the spike amplitude. The criterion for a single unit was constant amplitude and waveform of the action potentials and an absolute refractory period of at least 2 ms. The large spikes of a unit were detected with a window discriminator (Dagan, WD-2), digitized (CED, Micro 1401), and recorded on a computer using CED Spike2 software; data was also recorded on VCR tape (Medical Systems, PCM4/8). Units with large oscillations of spontaneous activity were not used.

Data Analysis

The units presented in this study were selected from 54 single unit recordings that were presumed to be from mitral cells based on the depth in the MOB. In 5 cases, the unit was lost before activity could be recorded or the recording became unstable. In 8 cases, the location of the recording was uncertain either because a dye mark was not recovered or because histological evaluation of dye marks showed that a unit was recorded in the external plexiform or granule cell layer. In 13 cases, the unit could not be antidromically activated from the posterior piriform cortex. This left 29 units recorded from 15 different rats that were identified as mitral cells based on depth, reversal of the field potential, antidromic activation and for most a dye mark in the mitral cell body layer.

In 12 of the 29 units, 2 periods of spontaneous activity were recorded separated either by the recording of the antidromic activation, introduced artifactual electrical noise, or an

adjustment of the position of the recording electrode. There was no difference in the mean spontaneous activity between these separate recordings from the same unit (paired t tests: mean rate ($t = 1.57$, $df=11$, $P = 0.14$); CV of interspike interval ($t = 0.69$, $df=11$, $P = 0.50$)). The period used to represent a unit with 2 periods was selected at random, and data from only that period is presented in Table 1.

For each mitral cell unit, the average latency between posterior piriform cortex stimulation and the antidromic action potential in the MOB was calculated for 5-10 stimuli. Recordings of spontaneous activity lasted for a mean of 540 s (range 100 -1243 s). For each unit we calculated the mean rate, the mean interspike interval, the standard deviation of the intervals, and the coefficient of variation (CV) of the interval.

We conducted a hierarchical cluster analysis using the latency, mean rate, and coefficient of variation (CV) in the interspike interval to test whether two distinct subpopulations of mitral cells could be demonstrated. Following cluster analysis, a *post hoc* multivariate analysis of variance (MANOVA) was performed to determine if there were significant differences among the groups and which variables contributed to the differences. To explore whether the pattern of spike discharge in mitral cell units differed between the two clusters, each unit was evaluated with an autocorrelation function. The bin/lag time was set at 2 ms to avoid a large negative correlation during the first lag due to the absolute refractory period. We examined the autocorrelation for a lags up to 400 ms. All statistical analyses were conducted using SPSS 17.0.

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Two subclasses of mitral cells were distinguished based on spontaneous activity

Group A mitral cells have a higher mean rate of action potentials

Group A mitral cells tend to fire in bursts

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Fig. 1.

Two examples of single unit spontaneous activity. The unit shown in **A** was recorded for 640 s; the rate of action potentials (left panel) ranged from 8 to 39 spikes/s with a mean of 21.7spikes/s. The inset (above) shows the timing of individual spikes for 2 s during the recording; the spike pattern consisted of occasional bursts of 3-4 spikes with very short interspike intervals separated by longer intervals. The pattern for the complete recording duration is illustrated in the interspike interval histogram shown in the right panel where almost 30% of the interspike intervals were 4-5 ms. **B** shows another unit recorded for 311s; the rate of action potentials (left panel) ranged from 1 to 14 spikes/s with a mean of 7.4 spikes/s. The inset (above) shows individual spikes for 2 s during the recording; there is little bursting. The interspike interval histogram (right panel) shows a relatively even distribution of intervals between 5 and 100 ms.

Fig. 2. Comparison of the latency, mean rate and CV of interspike interval for two groups of mitral cells identified by hierarchical cluster analysis. The means (GroupA versus Group B) are latency: 6.18 ± 2.89 versus 5.08 ± 1.51 ms; mean rate: 28.94 ± 4.7 versus 12.87 ± 3.34 spikes/s; CV of interspike interval 1.40 ± 0.32 versus 1.45 ± 1.33 . Only the difference in mean rate is significant (see text).

Fig. 3. Autocorrelation functions for different mitral cell types. The top panel shows two random “fast” mitral cells (the first two classified in the data set) and the mean for that group (solid line). The bottom panel shows two “slow” mitral cells and the mean for that group of cells.

Table 1

Group assignments for mitral cells. 29 single units showing the duration of the recording, the latency for an antidromic action potential following stimulation of the posterior piriform cortex, the mean rate, mean interspike interval, standard deviation of the intervals (SD), the coefficient of variation (CV) in interspike interval, and the group assigned by cluster analysis.

Unit ID	Duration (s)	Latency (ms)	Mean rate (spikes/s)	Mean interval (s)	SD	CV	SD/mean	Group
N110100	800	11.4	25.30	0.04	0.06	1.56		A
N122000d	1243	4.9	18.67	0.05	0.07	1.24		B
N010401e	390	5.3	9.96	0.10	0.10	0.96		B
N011801d	620	3.1	13.19	0.08	0.09	1.23		B
N011801f	191	3.7	26.40	0.04	0.04	1.04		A
N011801i	910	4.5	17.13	0.06	0.07	1.15		B
N011801k	729	6.7	29.65	0.03	0.04	1.33		A
N020601e	640	4.4	21.68	0.05	0.06	1.21		A
N021301d	640	3.5	34.62	0.03	0.06	2.01		A
N021301g	630	2.9	14.53	0.07	0.07	0.97		B
N021501a	618	6.2	13.32	0.08	0.09	1.15		B
N021501e	1000	6.4	12.25	0.08	0.13	1.56		B
N022001b	100	4.6	35.01	0.03	0.05	1.64		A
N022001g	715	4.9	12.20	0.08	0.12	1.48		B
N022001j	673	7.6	12.29	0.08	0.08	0.95		B
N030101b	266	4.6	15.55	0.06	0.07	1.01		B
N030101e	182	9.5	31.89	0.03	0.04	1.39		A
N040501a	247	4.5	15.26	0.07	0.07	1.14		B
N040501b	215	5.8	8.00	0.13	0.11	0.89		B
N040501d	458	7	16.72	0.06	0.06	1.09		B
N041201a	607	5.8	14.90	0.07	0.07	1.00		B
N041201e	311	6	7.42	0.13	0.12	0.87		B
N041701b	312	2	12.43	0.08	0.10	1.28		B
N041901b	301	2.6	14.29	0.07	0.09	1.23		B
N042401a	311	4.8	4.60	0.22	1.55	7.11		B
N042401f	322	5.5	26.95	0.04	0.04	1.06		A
N050301c	226	7.4	11.30	0.09	0.20	2.24		B

Unit ID	Duration (s)	Latency (ms)	Mean rate (spikes/s)	Mean interval (s)	SD	CV	SD/mean	Group
N050301c	250	5	13.22	0.08	0.07	0.93		B
N050301g	796	5.3	13.06	0.08	0.08	1.05		B