Neuronal Density Determines Network Connectivity and Spontaneous Activity in Cultured Hippocampus

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Ivenshitz M, Segal M. Neuronal density determines network connectivity and spontaneous activity in cultured hippocampus. J Neurophysiol 104: 1052–1060, 2010. First published June 16, 2010; doi:10.1152/jn.00914.2009. The effects of neuronal density on morphological and functional attributes of the evolving networks were studied in cultured dissociated hippocampal neurons. Plating at different densities affected connectivity among the neurons, such that sparse networks exhibited stronger synaptic connections between pairs of recorded neurons. This was associated with different patterns of spontaneous network activity with enhanced burst size but reduced burst frequency in the sparse cultures. Neuronal density also affected the morphology of the dendrites and spines of these neurons, such that sparse neurons had a simpler dendritic tree and fewer dendritic spines. Additionally, analysis of neurons transfected with PSD95 revealed that in sparse cultures the synapses are formed on the dendritic shaft, whereas in dense cultures the synapses are formed primarily on spine heads. These experiments provide important clues on the role of neuronal density in population activity and should yield new insights into the rules governing neuronal network connectivity.

I N T R O D U C T I O N

The density of synapses among neurons in the brain varies considerably during development. Variations in connectivity contribute to circuit refinement and efficacy of connections (Colman and Lichtman 1993; Lichtman and Colman 2000). For example, in the cerebellum, there is an overproduction of synapses at early developmental stages and postsynaptic targets receive input from multiple climbing fibers. Later in development most synapses made from climbing fibers onto Purkinje cells disappear (Kano et al. 1995) and only one axon, which has a high probability of multivesicular release, maintains input onto a given postsynaptic cell (Hashimoto and Kano 2003). In the visual system, thalamocortical axons disconnect from cortical layer IV cells (Gordon and Stryker 1996) and receptive fields of neurons in the binocular zone become sharper and are dominated by one eye as the visual system matures (Hubel et al. 1977). The reinnervation of connections undergoes a significant rearrangement of axons even after the initial connections are formed (Sur et al. 1984), which may contribute to the sharpening of receptive fields (Tootle and Friedlander 1989). Chen and Regehr (2000) showed a 50-fold increase in synaptic strength in the remaining synapses. It was suggested that α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) insertion allows for the increase in synaptic strength, shown by an increase in AMPAR quantal size and number of release sites, but it could also result from a presynaptic increase in number of release sites.

The evidence presented by these and other groups suggests that a common denominator of neural and synaptic development is that input elimination sharpens neural response during maturation; as the number of presynaptic partners is reduced, the synaptic strength in the remaining cells is increased. Although this concept has been proposed some time ago (Liu and Tsien 1995), the relationship between synaptic density and strength has not been studied systematically under controlled conditions. The dissociated culture of primary central neurons provides a convenient test system for analysis of the role of network density on connectivity among neurons.

Spontaneous network activity plays an important role in the maturation of neural tissue and has been found in most central neuronal networks examined both in vivo and in vitro (Khazipov et al. 2001; Minlebaev et al. 2007). Despite extensive documentation of spontaneous network activity, little has been done to delineate the elementary rules that govern the formation and spread of network activity. One such study examined the role of network size on its activity (Wilson et al. 2007a), but whether there is a trigger zone for such activity and how it spreads have not been studied systematically, except for some unique test systems (Feinerman et al. 2007).

In the present study we analyze morphological and electrophysiological differences among neurons grown in sparse, medium, and high-density cultures. We found that spontaneous activity is largely dependent on the number of neurons comprising a network. Importantly, we show that the strength of connections among neurons is inversely correlated with the number of available presynaptic partners, revealing that the neurons optimize their synaptic strength in response to cues in their surrounding network size. Furthermore, synapses are localized primarily on the dendritic shaft in sparse cultures, whereas in dense cultures synapses are made on dendritic spines, raising the possibility that shaft synapses may have a more significant impact on the neuron than previously assumed.

M E T H O D S

Cultures

Animal handling was done in accordance with the guidelines published by the Institutional Animal Care and Use Committee of the Weizmann Institute and with the Israeli National Guidelines on animal care. Cultures were prepared as detailed elsewhere (Goldin et al. 2001). Briefly, rat pups were decapitated on the day of birth (P0), their brains removed and placed in a chilled (4°C), oxygenated Leibovitz L15 medium (Gibco) enriched with 0.6% glucose and gentamycin (20 μg/ml; Sigma). Hippocampal tissue was mechanically dissociated after incubation with trypsin (0.25%) and DNAase (50 μg/ml) and passed to the plating medium consisting of 5% heat-
inactivated horse serum (HS), 5% fetal calf serum, and B-27 (1 μl/ml) prepared in minimum essential medium (MEM) Earl salts (Gibco), enriched with 0.6% glucose, gentamicin (20 μg/ml), and 2 mM glutamate (enriched MEM). About 50K, 100K, or 500K cells (sparse, medium, and dense) in 1 ml medium were plated on 12 mm round cover glasses in each well of a 24-well plate, onto a hippocampal glial feeder layer, which was grown on the glass for 2 wk prior to the plating of the neurons (Papa et al. 1995). Cells were allowed to grow in the incubator at 37°C, 5% CO2 for 4 days, at which time the medium was changed. Transfection was carried out on 7 DIV cultures.

Electrophysiology

Hippocampal cultures at 10–14 days in vitro (DIV) were transferred to a recording chamber placed on the stage of an inverted Olympus IX70 microscope and washed with a standard recording medium, containing (in mM) NaCl, 129; KCl 4; CaCl2, 2; MgCl2, 1; glucose, 10; and HEPES, 10 (pH 7.4, 320 mM). Neurons were recorded with patch pipettes containing (in mM): K-gluconate, 136; KCl, 10; NaCl, 5; HEPES, 10; ethylene glycol-bis (beta-aminoethyl ether) N,N′,N′,N′-tetra-acetic acid (EGTA), 0.1; Na-GTP, 0.3; Mg-ATP, 1; and phosphocreatine, 5 (pH 7.2 with a resistance in the range of 5–10 MΩ). Junction potentials of about 10 mV were left uncompensated. The recorded cells had a membrane potential (Vm) of at least −55 mV and their monitored series resistance and capacitance were not changed significantly throughout the recording session. Signals were amplified with MultiClamp 700B and recorded with pClamp9 (Axon Instruments, Foster City, CA).

To study synaptic connections, dual whole cell recordings were conducted. Minimal DC current required to evoke an action potential was injected into the presynaptic neuron in current-clamp mode; 36 postsynaptic inward current responses to the afferent stimulation were recorded in voltage-clamp mode at 10 s intervals and subsequently averaged.

When recording spontaneous miniature excitatory postsynaptic currents (mEPSCs), 0.5 μM tetrodotoxin (TTX) was added to the medium and neurons were clamped at −60 mV. For recording of miniature inhibitory postsynaptic currents (mIPSCs), 0.5 μM TTX, 20 μM 6,7-DNQX (dinitroquinoxaline-2,3-dione), and 50 μM APV (2-amino-5-phosphonovaleric acid) were added to the recording medium, a CsCl-based intracellular solution was used, and cells were clamped at −60 mV.

Transfection

A lipofectamine 2000 mix was prepared at 1 μl with 50 μl Opti-MEM per well, and incubated for 5 min at room temperature. This was mixed with 1 μg green fluorescent protein (GFP) and 1 μg polysynaptic density protein 95 (PSD95)-DsRed plasmids per well in 50 μl Opti-MEM and incubated for 15 min at room temperature. The mix was then added to the cultures for 3–5 h until medium was changed. Transfection was carried out on 7 DIV cultures.

Morphological analysis

Cultures were washed with a standard recording medium and placed in a recording chamber. For normal morphological analysis, calcein was injected into the neurons through the recording patch pipette. The chamber was placed on the stage of an inverted Zeiss confocal laser-scanning microscope (LSM-510). High-resolution thin optical sections of the cells were obtained. Off-line analysis included three-dimensional reconstruction of the neurons and dendrites and image analysis was conducted using LSM image analysis software. Sholl analysis, which measures dendritic length, number of branch points, and dendritic tortuosity, was then carried out by counting the number of dendritic crossings at concentric spherical surfaces drawn around the soma. Axons, which have a constant thin diameter regardless of the distance from the soma, were not counted.

Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 30 min, blocked in appropriate serum, and stained overnight with the primary antibody (mouse anti-NeuN, 1:1,000; rabbit anti-glutamic acid decarboxylase [GAD], 1:2,000; donkey anti-PSD95, 1:2,000; mouse anti-synaptophysin, 1:1,000) and then with Alexa-conjugated fluorescent secondary antibodies (1:200). The number of total cells minus the number of GABAergic cells was taken to represent the number of glutamatergic cells in culture.

Calcium imaging and analysis

Cultures were incubated for 1 h at room temperature with the standard recording medium containing 2 μM Fluo-4/AM (Invitrogen/Molecular Probes, Carlsbad, CA). Cells were imaged thereafter on the stage of an upright Zeiss Axioscope microscope, equipped with a Till Photonics light source and a Sensicam charge-coupled device camera (PCO Imaging). High temporal-resolution images (100–200 frames/s) were taken with an IXON ANDOR camera, linked to the electrophysiological recording system, as before. Each field of the culture (190 μm2) was imaged for 2 min and four to six fields were imaged for each experimental condition. Fluorescence levels at 520–550 nm to illumination at 488 nm were recorded. Axon Imaging Workbench (AIW V5, Axon Instruments) was used for data acquisition and computer control. Regions of interest (ROIs) were marked around neuronal somata, resulting in a set of average fluorescence level graphs for each ROI at any given time point. Parametric analysis was conducted with a Matlab program (Cohen et al. 2008). The results are presented as dF/F.

Statistical analysis

The quantified data were analyzed by ANOVA using SPSS software. Post hoc comparisons were made using Tukey’s paired comparisons.

RESULTS

The number of neurons counted in the culture dish at 2 wk after plating is presented in Fig. 1. This number is substantially lower than originally plated partly because, at the time of plating, it is impossible to separate between the glia and neurons and the latter group of cells is highly sensitive to mechanical insults and many neurons could presumably have died in the process of plating. In addition, a significant portion of the cells adhere to the periphery of the plastic and not on the cover glass itself. Altogether, when we counted the number of neurons 3 days after plating, this number was already substantially lower than the cell count at the plating (Supplemental Fig. S2), indicating that the cells do not die over the course of the experiment, but right after plating: once they survive 3 days in the dish, most of them survive 2–3 more weeks in the dish. Counting neurons stained with the NeuN antibody showed that the sparse cultures contained 14 ± 2 neurons/mm2, the medium density cultures 25 ± 2 neurons/mm2, and the dense cultures contained 48 ± 2 neurons/mm2 $\left( F_{1,221} = 45.55, P < \right.$

1 The online version of this article contains supplemental data.
Recouting the cultures using the nuclear DAPI stain in addition to the staining for NeuN gave similar results (9 and 53 neurons in the sparse and dense cultures, respectively) (Supplemental Fig. S2). For clarity, the cultures will be referred to by their density as sparse, medium, and dense.

GAD immunohistochemistry showed that the percentage of inhibitory neurons did not change across the different cultures and remained at roughly 35% \( F_{(2,21)} < 1, n = 8 \) (Fig. 1, C and D), indicating that it is unlikely that the culture contains a select population of neurons with higher survival rate.

Intracellular calcium variations reflecting network activity were monitored in the three culture types. All cultures expressed spontaneous activity, but the sparse cultures exhibited fluctuations that were significantly different from their dense counterparts. In sparse cultures, the neurons expressed larger bursting activity with longer durations and long intervals between bursts. On the other hand, in dense cultures, a more active and robust pattern of activity was seen, which consisted of higher frequency and less synchronized bursts (Fig. 2). The differences among the three culture groups were highly significant. A significant change in burst amplitude for culture density was found \( F_{(2,33)} = 4.21, P < 0.02 \). Post hoc analysis revealed a larger burst amplitude in the sparse cultures (1.28 ± 0.23), compared with 0.63 ± 0.05 in the dense cultures (Fig. 2C). A similar trend was found with the burst width \( F_{(2,36)} = 7.25, P < 0.002 \). The average burst width in the sparse cultures was 10.46 ± 1.93 s, compared with 5.58 ± 0.23 and 4.19 ± 0.13 s in the medium and dense cultures, respectively (Fig. 2D). As indicated, sparse cultures exhibited a slower bursting pattern than did the more dense cultures \( F_{(2,36)} = 91.60, P < 0.0001 \), with 4.23 ± 0.8, 8.53 ± 0.8, and 21.90 ± 1.1 bursts per 2 min in the sparse, medium, and dense cultures, respectively (Fig. 2E).

These differences could reflect differences in calcium uptake and removal, but could also result from genuine differences in firing patterns between the neurons under the different conditions. To address this possibility more directly, current-clamp recordings were made from similar neurons, to reveal a trend similar to that seen with calcium imaging in populations of neurons. That is, in sparse cultures there were more bursts that were separated by long quiet periods. In dense cultures, there were more single action potentials that occurred at higher frequencies (Fig. 2B).

The difference in spontaneous network activity among the three types of cultures may result from differences in the strength of connectivity among the participating neurons. To examine this possibility we recorded simultaneously from randomly selected pairs of neurons in a dish. An action potential was evoked in a presynaptic neuron in current-clamp mode and the postsynaptic current was recorded in the second neuron of the pair in voltage-clamp mode at \(-60 \text{ mV}\). A significant decrease in evoked EPSC amplitude as the number of neurons in the network increased was noted \( F_{(2,23)} = 6.45, P < 0.006 \). The average EPSC amplitude in the sparse culture was 82.67 ± 14.96 pA, compared with 39.96 ± 10.19 and 22.94 ± 3.46 pA.
in the medium and dense cultures, respectively (Fig. 3, A and B). In addition, the strength of inhibitory connections was also inversely correlated with the number of potential partners in the network. The average evoked IPSC amplitude in the sparse culture was significantly larger than that in the dense culture (37.43 ± 8.86 vs. 14.97 ± 2.88 pA; *P* < 0.05, Fig. 3, C and D). Importantly, a larger proportion of cells was connected by an excitatory or inhibitory synapse in the sparse networks than in the dense ones. In the sparse cultures, 30.4% of neurons had a detectable synapse, with an adjacent neuron within a sphere of about 150 μm (7 of a total of 23 pairs); 22.72% in the medium (5 of 22); and only 17.50% in the dense cultures (7 of 40). These results indicate that both the number and strength of connections were inversely correlated with the network size.

The difference in evoked activity between the sparse and dense cultures can reflect a difference in one of several mechanisms; on the presynaptic side, an increase in the number of axon terminals or release sites and boutons impinging on a single cell in the sparse cultures, an increased release probability, or increased vesicle content of neurotransmitter; on the postsynaptic side, an increase in postsynaptic receptor number or function. To decipher which of these potential candidates might be responsible for the inverse correlation with synaptic strength, mEPSCs and mIPSCs were recorded. Surprisingly, neither a difference in amplitude \(F_{(2,16)} < 1\) for mEPSCs and \(F_{(2,16)} = 1.61, P > 0.23\) for mIPSCs nor frequency \(F_{(2,46)} < 1\) for mEPSCs and \(F_{(2,16)} < 1\) for mIPSCs was found. The average mEPSC amplitudes were 20.8 ± 1.33, 22.54 ± 4.3, and 21.10 ± 1.8 pA for the sparse, medium, and dense cultures, respectively (Fig. 4A). Their frequencies/min were 37.67 ± 12.1, 35.18 ± 12.5, and 38.32 ± 7.8. The average mIPSC amplitudes were 21.73 ± 2.6, 27.51 ± 4, and 19.26 ± 1.7 pA for the sparse, medium, and dense cultures, respectively (Fig. 4B). Their frequencies/min were 43 ± 12.3, 41 ± 9.7, and 22.25 ± 11.3, respectively.

The lack of any significant difference in miniature synaptic currents among the cultures indicates that the postsynaptic sensitivity to glutamate or γ-aminobutyric acid (GABA) did not differ in the three culture sizes. These results also indicate that the same number of active terminals is present in the sparse network in fewer neurons, such that activation of such a neuron produces larger synaptic currents and is able to drive the postsynaptic cell better than in the dense network. This assumption was tested by combining electrical recording and stimulation of single cells with high temporal resolution imaging of calcium variations in a population of adjacent neurons (Fig. 5, A–C). In sparse networks, stimulation of one neuron led to bursting of all or most other neurons in the field with a short (3–5 ms) synaptic delay. No such bursts could be evoked in the dense networks studied, even when the recorded neuron was depolarized to discharge several action potentials (Fig. 5D, 5–10 cultures each).

**FIG. 2.** Sparse networks exhibit larger and longer calcium bursts that do dense networks. A, left: representative images and continuous records of imaged neurons loaded with Fluo-4 AM at 3 plating densities. Scale bar: 1 arbitrary fluorescence unit, 10 s (right). B: representative current-clamp recordings of spontaneous activity in the 3 culture conditions. Scale bar: 20 mV, 5 s. C: summary of burst amplitude among the different cultures. D: average duration of the spontaneous bursts. E: averaged burst frequency (per 2 min) among the different densities. Numbers in bars indicate the number of fields taken for analysis.
Morphological differences among neurons grown in different network densities were then analyzed. Calcein was injected into randomly selected cells through a patch pipette. After several minutes the pipette was carefully withdrawn and the cells were imaged with a confocal microscope. Sholl analysis revealed a striking difference between the neurons in the different culture conditions between 40 and 140 μm away from the soma \( F(2,25) = 3.90 \) to \( P < 0.001 \). Post hoc Tukey comparisons revealed a larger number of dendritic crossings in the dense than that in the sparse cultures. For example, at a distance of 100 μm from the soma, a typical neuron in the dense cultures branched 29.80 ± 3.32 times.

**FIG. 3.** Neurons in sparse networks exhibit stronger synaptic connections. **A:** typical recording of an evoked action potential in the presynaptic neuron (bottom, recorded in current clamp) and the postsynaptic excitatory postsynaptic current (EPSC, top, recorded in voltage clamp, \( V_m = -60 \) mV) in the different culture densities. **B:** averaged amplitudes of the evoked EPSCs among the different cultures. **C** and **D:** same as in **A** and **B**, but for inhibitory postsynaptic currents (IPSCs).

**FIG. 4.** Amplitudes and frequencies of miniature (m)EPSCs and mIPSCs are not different among the different culture densities. **A:** mEPSCs. Sample traces of mEPSCs recorded at \(-60 \) mV, in 3 cells in the different density cultures, and their averaged (i) frequency per minute and (ii) amplitudes. **B:** same as in **A**, but for mIPSCs.
whereas the sparse neuron had only 12.30 ± 1.61 branch points (Fig. 6B). It also appears that the dendrites of neurons in the sparse cultures traveled a farther distance away from the soma than did their dense counterparts, indicating that they are perhaps searching more for presynaptic input. Whereas the sparse neuron’s dendrites branched 4 ± 2.63 times at 380 μm away from the soma, the dense neurons had no branch points at all.

Another striking difference between the cultures was in the density of dendritic spines. Neurons in dense cultures had a higher density of spines compared with that in the sparse cultures \(F_{(2,24)} = 7.02, P < 0.004\). The average number of spines in the sparse cultures was 0.13 ± 0.06/μm dendrite, compared with 0.19 ± 0.02 and 0.22 ± 0.02 in the medium and dense cultures (Fig. 6C). There was no change in spine length \(F_{(2,28)} = 2.63, P > 0.09\), with average lengths of 1.57 ± 0.09, 1.35 ± 0.08, and 1.34 ± 0.06 in the sparse, medium and dense cultures, respectively (Fig. 6D). Given that the dense cultures grow more dendrites than the sparse cultures, it appears that their total number of dendritic spines is far larger than those of the sparse culture.

The above-cited data demonstrated that neurons in sparse cultures have significantly fewer spines than the dense neurons; moreover, the evoked synaptic current is larger among the sparse neurons. Extrapolating from these two data sets, it would seem that the synapses in the sparse cultures are made on the shaft, whereas those made onto dense culture neurons are made on the dendritic spine. Taking this a step further, it can also be suggested that shaft synapses are stronger and produce a large postsynaptic current that do spine synapses. To study the location of synapses in the different cultures, neurons in the sparse and dense cultures were transfected with a GFP-PSD95 plasmid, and the number and location of PSD95 puncta were studied (Fig. 6, E and F). In sparse neurons, 89.8 ± 7.23% of puncta were on the dendritic shaft, with an average of 2.17 ± 1.27 puncta/50 μm dendrite length, compared with 0.33 ± 0.2 puncta on spines heads. In contrast, the majority of synapses in the dense neurons (71.7 ± 11%, n = 5 cells, \(P < 0.009\)) were made onto the dendritic spine (5 ± 1.4 puncta) compared with 2.3 ± 1.5 on the shaft, indicating that although there were altogether more synapses in the dense neurons, the synaptic connections between neurons in the sparse cultures are much stronger than those in the dense cultures.

**Discussion**

When networks of cultured neurons are comprised of a relatively small number of elements, their spontaneous activity is characterized by large bursts separated by long quiet periods. In addition, the connectivity between these neurons is stronger than that among cells in a dense network; high proportions of interconnected cells are found and stimulation of one member of the network produces large synaptic currents in adjacent cells. Morphologically, neurons in a sparse network have fewer spines and a simplified dendritic tree, compared with neurons in the dense network. Thus the relationship between the morphological and synaptic properties and the emerging network activity is not trivial and straightforward.

Spontaneous network activity is conceived as one of the earliest and most fundamental processes to occur within a network, with significant implications in network plasticity and adaptability, and the subsequent maturation is assumed to play a role in the further construction of functional networks (Opitz, 2009).
Studies looking at the development of network activity in cortical and hippocampal dissociated cultures document activity after only a few days in culture (Chiappalone et al. 2006), even before the formation of synapses, and can extend for days to weeks in different species. In the developing hippocampus, giant depolarizing potentials (GDPs) play an important role in the generation of network activity and may be dependent on the depolarizing action of GABA (Ben-Ari 2002). In cortical neurons, the generation of spontaneous synchronous bursts was shown to be governed by the level of spontaneous presynaptic firing, by the degree of connectivity of the network, and by a distributed balance between excitation and recovery processes (Maeda et al. 1995). Some studies have discussed percolated subthreshold activity or trigger neurons, which drive the rest of the network (Feinerman et al. 2005), whereas others have shown that the dynamics of the activity is largely controlled by the fraction of endogenously active cells in the network; when no spontaneously active cells are present, networks are either silent or fire at a high rate; as the number of endogenously active cells increases, there is a transition to bursting and then to a steady firing at a lower rate (Latham et al. 2000 a,b). Whether the number of intrinsically spiking neurons differs between the sparse and dense networks and plays a role in determining the different network activities remains to be checked, our results indicate that in the dense network there might be several independent trigger zones, each of which is capable of triggering the entire network. In support of this, Yvon et al. (2005) previously showed that in cultures of dissociated spinal cord the number of intrinsically active neurons increases with network size. On the other hand, our inability to trigger a network burst by stimulating a single neuron may indicate that either these trigger cells are very sparse even in the dense network or that only a concerted activity of many cells is needed to trigger network activity. Another aspect of the network bursting is the long silent periods between bursts in the sparse culture. These can be caused either by activation of some slow-voltage/calcium-gated K channels or by activation of slow, GABAB receptors, although these alternate hypotheses can be easily tested. Other studies that looked at spontaneous network activity discuss the age and maturity of the culture as being important factors in the activity pattern. In cortical cultures, 3-day-old neurons show uncorrelated and sporadic activity, which is transformed to synchronized bursting pattern within a week of the establishment of a culture and that, at 30 days, the network exhibits a complicated nonperiodic, synchronized activity pattern with no later changes occurring (Kamioka et al. 1996). Others have argued that in vitro cultures show two steps of maturation, i.e., synaptic functional connectivity of the network and general moderate and stabilized correlated activity.
which served to elevate their own inhibition to accommodate in evoked EPSCs. This increase was explained by an in-
in the large cultures, which was accompanied by a decrease in mEPSC amplitudes, in large versus small networks (with similar study (Wilson et al. 2007b) reported a large difference in absolute number of synapses, there was a massive difference in the size of the synaptic response between the two conditions. This may indicate that the shaft synapses are not less functionally relevant than spine synapses.

In conclusion, changes in synaptic strength are apparent in the different density cultures is an interesting and important property of neuronal networks. This inverse relationship between synaptic strength and number of potential afferent input partners may be an important rule for neurons in a network, allowing them to compensate for increasing innervations and preventing toxicity and overexcitability, on one hand, or increasing the input to approach the spike-firing decision dynamic range, on the other. The data presented here lend experimental support to the hypothesis of homeostatic control (Davis and Bezprozvanny 2001), although the mechanisms that sense changes in ambient innervation and make adjustments accordingly still need to be further explored.

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