Different Mechanisms of Calcium Entry Within Different Dendritic Compartments

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Received 12 March 2001; accepted in final form 7 November 2001

Single, Sandra and Alexander Borst. Different mechanisms of calcium entry within different dendritic compartments. J Neurophysiol 87: 1616–1624, 2002; 10.1152/jn.00215.2001. From our experiments combining in vivo calcium imaging and electrophysiology on fly vertical motion-sensitive cells (VS-cells) during visual stimulation, we infer different mechanisms of calcium entry within different dendritic compartments; while in the main dendritic branches calcium influx from extracellular space takes place only via voltage-activated calcium channels (VACCs), calcium enters the dendritic tips through VACCs as well as nicotinic acetylcholine receptors (nAChRs). Consequently, neuronal nAChRs of insects have to be assumed to be permeable to some extent for calcium under in vivo conditions.

INTRODUCTION

In vertebrates, neuronal nicotinic acetylcholine receptors (nAChRs) belong to the excitatory receptors being permeable to sodium, potassium, and, as has been demonstrated in a wealth of literature in the last several years, calcium (Adams and Nutter 1992; Castro and Albuquerque 1995; Mulle et al. 1992; Rathouz and Berg 1994; Sands and Barish 1991; Vernino et al. 1994). For insect nAChRs, only a few studies exist demonstrating their calcium permeability (Goldberg et al. 1999; Oertner et al. 1999; Van Eyseren et al. 1998). These studies were performed under in vitro conditions. Thus the calcium permeability of nAChRs described there cannot be automatically transferred to the in vivo situation, especially when the results were derived primarily from somatic nAChRs, the function of which is still not clear in insects. Our experiments presented in the following are aimed to close that gap, providing evidence that both receptor types become co-activated during preferred direction and during null direction motion, though to different extents. 1) This arrangement of co-activated excitatory and inhibitory input synapses can simply explain the saturation of membrane potential observed when the pattern of increasing sizes are moved in front of the fly (Haag et al. 1992). The saturation level mirrors the mixture of equilibrium potentials of both excitatory nAChRs and inhibitory GABA A Rs.

In vitro studies of the fly brain have demonstrated the existence of two different receptor types on LPTCs: a nACh and a γ-aminobutyric acid-A (GABA A ) receptor (Brotz and Borst 1996). The density of input synapses is highest in dendrites of higher order (Haase et al. 1980). There is convincing evidence that both receptor types become co-activated during preferred direction and during null direction motion, though to different extents. 2) The co-activation of excitatory and inhibitory input synapses can also explain the phenomenon of gain control (Ikeguchi et al. 2004; Haag et al. 1992; Hausen 1982) where the saturation of membrane potential observed when the pattern of increasing sizes are moved in front of the fly (Haag et al. 1992). The saturation level mirrors the mixture of equilibrium potentials of both excitatory nAChRs and inhibitory GABA A Rs.

The study was performed on large visual interneurons of the blowfly Calliphora vicina residing in the posterior part of the third neuropile of the optic lobe called “lobula plate” (Hausen 1984). Branching out in an almost two-dimensional layer, these lobula plate tangential cells (LPTCs) can be filled with a calcium-sensitive dye and filmed during motion stimulation (Borst and Egelhaaf 1992; Egelhaaf and Borst 1995). There exist about 60 different LPTCs per hemisphere, and each of them can be uniquely identified by its anatomy and response properties (Hausen 1982, 1984; Hengstenberg et al. 1982). The most prominent characteristic is their direction selectivity in the electrical and the calcium response when processing visual motion information (Single and Borst 1998); they depolarize, sometimes superimposed by spike-like events (Haag and Borst 1996; Hengstenberg 1977), and show a calcium increase when motion is in the preferred direction (PD), and hyperpolarize and show an overall calcium decrease when motion is in the opposite direction (null direction, ND). In this study, only results obtained from vertical-sensitive cells (VS-cells, type 1-11) will be presented. Their preferred direction is downward; the null direction is upward. These cells are postsynaptic in the lobula plate (Hausen et al. 1980) and possess large receptive fields because their large dendrites spatially pool the signals of thousands of local presynaptic columnar elements in a retinotopic fashion (Borst and Egelhaaf 1992; Haag et al. 1992).

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brane potential always saturates at the excitatory equilibrium level during different image velocities. Moreover, blocking the inhibitory input (i.e., GABA<sub>A</sub> receptors) leads to an increased depolarization during PD motion (Single et al. 1997).

Recent voltage-clamp studies in vivo have revealed a class of noninactivating low-voltage-activated (LVA) calcium channels on LPTCs. Their activation curve is flat and rises fairly linearly between −60 and −20 mV membrane potential (Haag and Borst 2000). At rest, these channels are already activated by about 30%, resulting in a permanent calcium influx. HVA calcium channels have not yet been found in LPTCs. Moreover, no evidence exists for metabotropic receptors or the contribution of intracellular calcium stores to calcium signaling in LPTCs (Brotz and Borst 1996; Oertner et al. 2001). Similarly, the contribution of a sodium-calcium exchanger operating in reverse mode to calcium increases during depolarizations could also be excluded. This exchanger seems to be only involved in the fast removal of calcium ions from cytoplasm (Oertner et al. 2001). This leaves only VACCs and nAChRs as possible entry gates for calcium in the cytoplasm of fly visual interneurons. Assuming that calcium-permeable nAChRs are located in the fine dendritic branches of VS cells, the interplay between the calcium responses via nAChRs and VACCs is predicted to take the following form (Fig. 1): 1) During PD motion the calcium concentration increases in all compartments of the cells (Fig. 1, B and C) by the activation of both

![Diagram](https://example.com/diagram.png)

**Fig. 1.** A: schematic activation levels of nicotinic acetylcholine receptors (nAChRs), γ-aminobutyric acid-A receptor (GABA<sub>A</sub>Rs), and postsynaptic membrane potentials during visual motion stimulation at optimal velocity along the preferred (PD) and the null direction (ND) of the cell. B: predicted calcium changes in the axon. Because no nAChRs are present, the calcium level is completely determined by the membrane potential through the activity of voltage-activated calcium channels (VACCs). C: predicted calcium changes in the dendrite. Here, the calcium level is jointly determined by the activity of VACCs and nAChRs: during PD motion, both voltage-dependent and nicotinic components increase; during ND motion, the voltage-dependent component decreases, while the nicotinic increases. Depending on the relative strength of both components, this might lead to a small increase of calcium during ND motion. D, E, F: the same as A, B, C, respectively, during motion stimulation at suboptimal velocity.
VACCs (throughout the cell) and nAChRs (highest in dendritic tips). 2) During ND motion the change in calcium concentration depends on the relative amount of hyperpolarization of membrane potential (shutdown of VACCs and, therefore, decrease of calcium level) and on the activation of calcium-permeable nAChRs (increase of calcium level) caused by the low-direction selectivity of the input elements (Fig. 1A). Because nAChRs are inhomogeneously distributed over the nerve cell membrane, this implies that the changes in calcium concentration will depend on the exact location within the neuron. In the axon and main dendrites (Fig. 1B), where the density of postsynaptic sites is low or even zero (Hausen et al., 1980), the calcium concentration decreases (shutdown of VACCs). At the dendritic tips (Fig. 1C), where synapse density is highest (Hausen et al. 1980), it may slightly increase (shutdown of VACCs, overcompensated by simultaneous activation of nAChRs). In the fine dendrites between the dendritic tips and the main branches, where nAChRs density is assumed to be intermediate, the calcium response should remain unchanged because the decreased calcium influx due to VACCs closing is just counteracted by the increased calcium influx through nAChRs. 3) As outlined above, the inputs to LPTCs show a weak direction selectivity, resulting in a co-activation of nAChRs and GABA_ARs during motion in preferred and null direction (Single et al. 1997). Since the activation ratio also depends on the image velocity also (Fig. 1, and Fig. A and D). The exact response of the calcium signal in the dendrites is therefore predicted to depend on the image velocity also (Fig. 1, C and F). All these predictions were tested in the experiments presented below.

METHODS

Preparation and stimulation set-up

Female blowflies (C. vicina) were prepared as described previously (Borst and Haag 1996) and mounted such as to look down onto a screen located 10 cm underneath them. They were stimulated by a moving stripe pattern that was produced by a rotating grating drum illuminated from the inside by an arc lamp, the image of which was projected to this screen. The square wave grating had a spatial wavelength of 26°, a mean luminance of 17.7 cd/m², and a contrast of 92%. Unless stated otherwise, the velocity of the moving pattern was 78°/s, corresponding to a temporal frequency of 3 Hz.

Electrical recording

Electrodes were pulled on a Brown Flaming micropipette puller (P-97, Sutter Instruments) using thin-wall capillaries with a diameter of 1 mm (GC100TF-10, Clark). When filled with 2 M KAc/0.5 M KC1/8.8 mM Ca-Green (Molecular Probes), they had resistances of about 30–40 MΩ. The output signal of a SEC-10L amplifier (NPI Electronics) operated in the discontinuous current clamp (switching frequency 20 kHz) or bridge mode was fed to a PC 486 via a 12 bit A/D converter (CIO-DAS16, Computer Boards) at a sampling rate of 3 kHz and stored to hard disk. The motor control unit for the rotating A/D converter (CIO-DAS16, Computer Boards) operated in the discontinuous current clamp (switching on the image velocity also (Fig. 1, and Fig. A and D). The exact response of the calcium signal in the dendrites is therefore predicted to depend on the image velocity also (Fig. 1, C and F). All these predictions were tested in the experiments presented below.

Optical recording

We used an upright epifluorescent microscope (Axioptec Vario, Zeiss) with Epiplan 10×/0.2 and Epiplan LD 50×/0.5 objectives (Zeiss) that were aligned to the backside of the opened head capsule of the fly. Calcium imaging was performed with filter set number 9 from Zeiss (excitation filter: BP 450–490 nm; beamsplitter: 510 nm; barrier filter: BP 516–565 nm) and a 12-bit CCD camera (PXL, Photometrics) connected to a Power-Mac (Apple). Unless stated otherwise, images were taken at 1 Hz at 128 × 128 pixel resolution. They were evaluated using the IPlab software (Scianalytics). Fluorescence images were expressed as relative changes of fluorescence (Δf/f) with respect to the second image of each series. To correct the Δf/f images for bleach, an unstained control area away from the cell was defined. The average fluorescence value of that area was subtracted from each pixel within the image. In the figures, the calcium dynamics are either expressed by a false-color code, warm colors representing an increase of calcium concentration, cold colors representing a decrease of calcium concentration, or by Δf/f time courses. The latter were obtained by averaging the pixel values within a given area in each image of the Δf/f image series.

Model simulations

Simulations were performed using a biophysically realistic compartmental model of a VS1-cell. The anatomy of the model cell was obtained from 3D reconstructions of cobalt-stained material as described previously (Borst and Haag 1996); anatomical data files of digitized LPTCs can be downloaded from http://nature.berkeley.edu/~borst/axel/projects/tanbase/download/download.htm. Using the “Nemosys” simulation software (Eeckman et al. 1994), all passive and active membrane properties were set as specified in Haag et al. (1997). In addition, we modeled a Ca current, distributed homogeneously over the whole cell, of the form I_Ca(t) = [V(t) − E_Ca] · g_{Ca} · h_Ca with g_{Ca} = 0.2 mS/cm² and E_Ca = 100 mV. m, and h, were of the form f(V) = 1/(1 + exp((MidV − V)/slope)) with MidV_Ca = 11 mV, slope_Ca = 8 mV, MidV_h = −3 mV, slope_h = −10 mV; m and h followed m_Ca and h_Ca by the first-order kinetic dx(t)/dt = [x(t) − x(t)/τ with τ_m = 7 ms and τ_h = 8 ms. Intracellular calcium concentration was calculated (de Schutter and Smolen 1998) as d[Ca]/dt = −I_Ca/2 Fv − β(Ca − [Ca]_{min}), with β set to 2 s⁻¹, corresponding to τ_v = 500 ms (Haag and Borst 2000), and [Ca]_{min} set to 100 nmol. The diffusion constant D was 6.10⁻⁶ cm²/s. The neuron was driven synaptically by two arrays of local motion detectors with the parameters as specified in Haag et al. (1999). 0.05% of the total current through nAChRs was modeled as being carried by calcium. The sine grating stimulating the local motion detectors had a spatial wavelength of 26°, a vertical extent of 64°, and an effective contrast of 3. When activated, it was moving at 78°/s.

RESULTS

In a first series of experiments we tested whether the calcium signals during ND motion indeed differ across the cell as predicted. For that we presented PD and ND motion to the fly and observed the VS1-cell response in different cellular compartments with a high magnification objective (50×), yielding a good spatial resolution of high-order dendrites. Figure 2 shows a typical experiment with two different protocols of PD and ND motion stimulation. The protocols on top consisted of 4 s stimulation of PD and ND motion with an intermittent period of 5 s where the pattern was at rest. The protocols on the bottom consisted of 2 s of PD motion followed by 12 s of ND motion, followed by 3 s of PD motion. This long duration of ND stimulation had the advantage that a steady-state level of calcium concentration was achieved. For technical reasons, electrophysiological data were not recorded in that experiment, but preceding recordings showed that, under these conditions, the cell hyperpolarized during ND motion. Averaging the calcium signal in four different dendritic areas (see raw fluo-
of the screen moving downward (PD), and after a pause of 9 s moved upward again (ND). The time courses represent the calcium responses at the axon and the dendritic tips during PD and ND motion. As with the moving grating in Fig. 2, all compartments of the VS1-cell showed an increase of calcium during PD motion. During ND motion, the calcium concentration in the axon and the main dendritic branches decreased, whereas it increased above resting level in the fine dendritic branches. All these results are in line with the predictions made above, thus supporting the hypothesis of calcium-permeable nAChRs in vivo.

To test whether the dendritic calcium levels depended on the image velocity, we displayed ND motion at two different velocities. Figure 4 shows the averaged results of such experiments performed on eight VS cells. When comparing ND motion at 2 (optimal) and 9 Hz (suboptimal) temporal frequency (corresponding to a velocity of 52 and 234°/s, respectively), the latter velocity led to a smaller hyperpolarization than the first (Fig. 4, bottom panels). The average electrical responses were around −7 mV at ND2/Hz and around −2 mV at ND9 Hz motion as compared with the resting potential. In the main dendrites, ND motion resulted in a calcium concentration decrease during motion at both velocities (Fig. 4, top panels, white bars). The exact amount of calcium decrease corresponded well to the extent of hyperpolarization in each case. In the dendritic tips, the situation was different. During ND2 Hz, the calcium signal was slightly negative, while during ND9 Hz motion, the calcium signal was positive (Fig. 4, top panels, black bars). Again, these results are in accordance with the predictions and provide additional evidence for the hypothesis that dendritic nAChRs are calcium permeable in vivo.

We further tested our hypothesis of the additive contribution of VACCs and nAChRs to the calcium response during PD motion by injection of hyperpolarizing current during PD motion (Fig. 5). To isolate the nicotinic current fraction VACC blockers, as Cd2+ for example, or low Na+ saline had been used before in the in vitro preparation of the fly tangential cells.
activity is located in the lower range of the activation curve (Haag and Borst 2000). Therefore when depolarization shifts the resting activation of the VACCs toward more positive values, the stronger decrease of calcium concentration caused by VACCs de-activation during ND motion should now outweigh the increase that is caused by activation of nAChRs. As a consequence, the calcium level should clearly decrease. In Fig. 6, the result of such an experiment is shown. As control, the calcium response of a VS1-cell to ND motion without electrical manipulation was recorded first (Fig. 6A). The cell showed a small hyperpolarization in its electrical trace, but the calcium response was nearly zero. When a depolarizing current of +5 nA was injected, the calcium level augmented (see arrow number 1, Fig. 6B). Starting of ND motion now led, in contrast to Fig. 6A, to a pronounced decrease of calcium concentration in the fine dendrites (see arrow number 2, Fig. 6B). These results demonstrate that the hyperpolarization occurring during ND motion de-activates VACCs, which are partially activated during resting conditions. This reduced calcium influx is counteracted by an increased calcium influx through nAChRs.

DISCUSSION

By presenting ND motion and observing the cellular fluorescence changes at high magnification, a spatially inhomogeneous calcium response was observed pointing to different cellular and even dendritic compartments. While the axon and the main dendrite showed a decreased calcium response, the dendritic tips exhibited elevated calcium levels. This profile could be explained by a spatially inhomogeneous distribution of different calcium-permeable channels, one class being voltage-gated, the other being transmitter-gated. Because current injection experiments have shown an overall increase of calcium level in the LPTCs, VACCs were concluded to be located throughout the cell. During PD motion they become activated; during ND motion they are shut down below their residual resting activity. Postsynaptic specializations have been found in high density in higher order dendrites (Hausen et al. 1980). It was furthermore shown that even during ND motion, excitatory input elements become activated, though to lesser extent than the inhibitory input elements (Single et al. 1997). If nAChRs were not calcium permeable, the calcium level should only depend on VACCs. If they were calcium permeable, the resulting calcium level during ND motion should result from the mix of activated nAChRs and de-activated VACCs. Depending on the density of the nAChRs, the calcium level could increase (more activated nAChRs than de-activated VACCs) or decrease (more de-activated VACCs than activated nAChRs). This is exactly what we have observed in VS cells; in the higher order dendrites the calcium level increased, pointing to more activated nAChRs than de-activated VACCs. In lower order dendrites and axon, the opposite was found to be true. The conclusion that nAChRs on dendrites in VS cells are calcium permeable is further supported by displaying different motion velocities using the different activation ratios of excitatory and inhibitory inputs. Presenting ND motion at a temporal frequency of 2 Hz, relatively more GABA\textsubscript{A}Rs and less nAChRs were activated than during 9 Hz. Therefore at 2 Hz, the calcium response should be less positive (or even negative,
FIG. 5. Demonstration of calcium-permeable nAChRs by combining PD motion stimulation and ramp-like hyperpolarizing current injection. Time courses of calcium and electrical signals and color-coded images of whole VS cell plus magnified insets of higher order dendritic branches showing the relative change of fluorescence in different cell compartments are presented. A: control—only PD motion is presented to the cell. B: simultaneous presentation of PD motion and ramp-like hyperpolarization. The color-coded image at the time of highest current injection is represented (see square).

FIG. 6. Combining ND motion and depolarizing current injection to portray the low activation of VACCs during rest. A: stimulation of VS1-cell by ND motion as control. B: stimulation of the same cell by ND motion under depolarizing current injection (+5 nA). If the activation of the VACCs is augmented by a preceding depolarization (arrow number 1), ND motion leads to a prominent decrease of calcium level by de-activating VACCs (arrow number 2).
Calcium responses in fine dendrites in detail

When carefully comparing the calcium responses in the fine dendrites during ND motion at 2 Hz in Figs. 2, 4, and 6, one can perceive differences. In Fig. 2, the calcium responses are above the calcium resting level; in Figs. 4 and 6 they are below or nearly zero. The exact value strongly depends on the precise dendritic location where the calcium signal has been averaged. Using 10× objectives, the spatial resolution is poorer and therefore not only the calcium signal in the very fine dendritic tips, but also in dendrites of lower order are averaged, resulting in a more negative calcium response than using the 50× objective where the spatial resolution is high. This fact might well explain why Egelhaaf and Borst (1995) also observed no calcium response during ND motion. They used a 10× objective and averaged the calcium signal in high- and lower dendritic branches. When examining the calcium responses during ND motion at the suboptimal velocities of 9 Hz (Fig. 4B) or 0.5 Hz (Fig. 3), the calcium signal in the fine dendrites even using 10× objectives is always above the resting level. This is in line with the explanations above. Using velocities above or below the optimal frequency of 2 Hz, the component of hyperpolarization is smaller and the component of activated nAChRs is bigger (compared with stimulation with velocity of 2 Hz), resulting in more positive calcium signals in fine dendritic branches.

Space clamp

An alternative explanation of the remaining calcium influx during hyperpolarization (Fig. 5B) could be that the cell is electrically not compact and, therefore, the hyperpolarizing current injected into the axon does not reach the dendritic tips of the cell. To explore this possibility, we performed simulation studies using a biophysically realistic compartmental model of a VS1-cell (Fig. 7). The anatomy of the cell (Fig. 7A) was obtained from 3D reconstructions of cobalt-stained material and its passive and active membrane parameters were derived from a series of current- and voltage-clamp experiments (Borst and Haag 1996; Haag et al. 1997). If such a compartmental cell is synaptically driven by an array of Reichardt detectors (Fig. 7A, right) that are stimulated by constant PD motion (first 2 s), both the electrical (Fig. 7B) and the calcium responses (Fig. 7C) rise. If additionally a hyperpolarizing current of −8 nA is applied (next 2 s), the calcium signal decreases and the electrical response is negative. This negative electrical signal for all cellular locations indicates that the hyperpolarizing current also reaches the dendritic tips. Based on these model simulations, the VS1-cell seems compact enough to perform such experiments as seen in Fig. 5. As is the case in the experiments of real VS1-cells, the calcium signal in the axon (black) decreases below the resting level, while in the dendritic tips (green) it also decreases but stays above the resting level.

Relative amounts of voltage versus transmitter-driven calcium

The experiment presented in Fig. 5 allows for a rough estimate of the ratio of calcium influx driven by VACCs and that driven by nAChRs. The calcium signal in the fine dendrite (blue) during PD motion and highest concomitant hyperpolarizing current injection amounts to about 30% of that during PD motion alone. Assuming that the calcium current via VACCs was completely eliminated leaving only the calcium current carried by nAChRs, we conclude that the ratio of VACCs carried calcium influx over nicotinic calcium influx during PD motion is about 2:1 under these conditions. This in vivo result perfectly matches the results obtained in the simulation studies (Borst and Single 2000). The calcium current, however, should be augmented in comparison to natural stimulation because the driving force for the calcium ions was increased by the imposed hyperpolarization.

While we activated nAChRs in vivo by visual stimuli, they can also be activated in vitro by iontophoretic stimulation with...
intracellular calcium and ATP concentration compared with in situ conditions due to their high sensitivity to the probably altered intracellular calcium and ATP concentration compared with in vivo, caused by the fact that the VACCs are not totally functioning, starting with the stress the cells suffer during preparation (leading to limited oxygen supply) and continuing with the extracellular ion composition that might differ from that of the fly hemolymph. All these factors can result in an augmented resting calcium concentration in the in vitro preparation leading to a partial inactivation of the VACCs. This might explain why the nicotinic component was found to be the dominant factor under in vitro conditions.

**Functional role of different calcium responses within the dendrite and the axon of the neurons**

Regarding the cellular distribution of calcium-permeable nAChRs and VACCs, two different functions of calcium signaling can be imagined. Obviously, in nerve terminals calcium is important for synaptic transmission. Here, only VACCs exist. These are activated to some extent during rest (Haag and Borst 2000). During PD motion, they become more strongly activated, leading to an increase of the calcium level. During ND motion, they become de-activated and shut off, leading to a decrease of calcium level (Single and Borst 1998). The existence of calcium channels with such characteristics in nerve cell ending is not so astonishing bearing in mind that the LPTCs continuously detect even the smallest changes of motion velocity and direction in the context of visual orientation behavior (Hausen 1982; Hengstenberg et al. 1982). This is optimally performed by the graded positive or negative electrical responses of the LPTCs (Haag and Borst 1997). These de- and hyperpolarizing responses can be synaptically transmitted in a graded way to postsynaptic nerve cells by this type of calcium channels that are found in the LPTCs. In dendritic tips, the same type of calcium current as in nerve terminals is found. Additionally, however, calcium-permeable nAChRs exist there, resulting in an increase of calcium level during PD as well as during ND motion. Thus the dendritic calcium signal depends less strongly on the direction of motion, but rather on the input activity, largely irrespective of its sign. Interestingly, a number of phenomena have been studied in fly LPTCs which share this characteristic and which have been previously attributed to columnar elements presynaptic to the tangential cell, e.g., a pronounced reduction in contrast gain after prolonged exposure to motion stimuli was found to be independent of the direction of the adapting stimulus (Harris et al. 2000) and locally confined (Maddess and Laughlin 1985). In a similar way, adaptation of the time constant of the velocity impulse response turned out to be directionally insensitive (Borst and Egelhaaf 1987). Our finding of a locally restricted dendritic calcium signal which is less dependent on the direction of motion hints to the possibility that such phenomena are produced within the LPTC’s dendrite by calcium starting second messenger cascades or acting on Ca-dependent K channels, the existence of which has been deduced indirectly from various experiments (Kurtz et al. 2000; Oertner et al. 2001; Single 1998).

We are grateful to H. Nguyen for excellent technical assistance and Dr. J. Haag for technical help and critically reading the manuscript.

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J Neurophysiol **VOL. 87 • MARCH 2002 • www.jn.org**


