Gating of single Shaker potassium channels in *Drosophila* muscle and in *Xenopus* oocytes injected with Shaker mRNA

(ion channel/patch-clamp method/protein function)

W. N. ZAGOTTA, T. HOSHI, AND R. W. ALDRICH*

Department of Neurobiology, Stanford University School of Medicine, Stanford, CA 94305-5401

Communicated by Charles F. Stevens, June 14, 1989

ABSTRACT The voltage-dependent gating mechanism of single A-type potassium channels coded for by the Shaker locus of *Drosophila* was studied by single-channel recording. A-type channels expressed in *Xenopus* oocytes injected with Shaker B and Shaker D mRNA exhibited gating and voltage dependence that were qualitatively similar to those of the native Shaker A-type channels from embryonic myotubes. In all three channel types the molecular transition rates leading to the first opening were voltage-dependent, whereas all transitions after the first opening, including inactivation, were independent of voltage. While these channels exhibit some quantitative differences in their transition rates that account for the observed differences in macroscopic currents, in all three cases the voltage dependence of the macroscopic currents is determined by a voltage dependence in the time to first opening. This gating mechanism is similar to that of the vertebrate voltage-gated sodium channel and, together with the sequence similarities in the S4 region of the proteins, suggests a conserved mechanism for activation and inactivation.

A-type potassium channels in neurons, muscle, and other cells contribute to spike repolarization, low-frequency repetitive firing, modulation of transmitter release and integration of synaptic input (1, 2). A class of A-type channels is coded for by the Shaker locus in *Drosophila* (3–6). The Shaker gene gives rise to a large family of transcripts, many of which can individually produce macroscopic A-type potassium currents when injected into *Xenopus* oocytes (7–12). The deduced sequences of Shaker protein contain a region, called S4, that is also present in the voltage-dependent sodium and calcium channels (3, 4, 8, 9, 13–19). This region has a highly conserved recurring motif of a positively charged amino acid at every third position with intervening hydrophobic residues. Current models for voltage-dependent gating favor a rotation or translation of S4 helices through the membrane as a prerequisite for channel opening (20–22). Outside of the S4 region, the Shaker sequences are not particularly similar to those of the sodium channel or calcium channel, although they seem to share a general topology of putative cytoplasmic, external, and membrane-spanning domains. The Shaker proteins also do not exhibit the 4-fold repeat of homology units of the sodium or calcium channel, leading to the idea that the Shaker channel is made up of an association of multiple Shaker subunits, which would be identical in the case of oocytes injected with single mRNA species, but perhaps composed of an aggregation of subunits coded by the different alternatively spliced messages in the native channels. In this report we address two questions. (i) Are there qualitative differences between the gating of native channels and those expressed in oocytes that suggest the inclusion of other gene products in the native channels? (ii) Is the voltage-

dependent gating of Shaker A-type channels similar to that of voltage-gated sodium channels even though the sequence similarity is essentially limited to the S4 and surrounding hydrophobic regions and the Shaker peptide contains only one S4 domain? We have examined the properties of single Shaker channels in *Xenopus* oocytes injected with Shaker B (ShB) and Shaker D (ShD) (7) mRNA and of native channels in cultured *Drosophila* myotubes.

MATERIALS AND METHODS

Myotube Culture. Cell cultures of *Drosophila melanogaster* myotubes were prepared according to the procedure of Seecof (23). Late-gastrula-stage embryos were collected and dechorionated in a 50% bleach solution for 1 min. Cells were then removed from the embryos with sharp micropipettes and dispersed onto untreated glass coverslips. The cells were allowed to differentiate for 10–15 hr at 26°C in a modified Schneider's medium containing 20% heat-inactivated fetal bovine serum and 8 milliunits of insulin per ml.

RNA and Oocyte Preparation. ShB and ShD cDNAs, cloned into the *EcoRI* site of the plasmid expression vector pSP72 (Promega), were obtained from the laboratory of L. Y. Jan and Y. N. Jan (Univ. of California, San Francisco, CA). The pSP72 plasmids with Shaker inserts were linearized with *HindIII* and used as templates for runoff transcription. Transcription reactions were conducted with 2–5 µg of template DNA, 500 µM NTPs, 500 µM m⁷G(5')ppp(5')G (a cap analog), 160 units of RNasin (Promega), and 40 units of T7 RNA polymerase in a standard transcription buffer. The template DNA was removed with DNase (RQ1 DNase, Promega), and RNA was purified by extractions with phenol/chloroform (1:1, vol/vol) and chloroform, ethanol-precipitated, and resuspended in 10 µl diethyl pyrocarbonate-treated water. Oocytes were injected with mRNA in a manner similar to that described previously (24). Female *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI) and maintained at room temperature (20–22°C). The frogs were anesthetized by immersing them in 0.1–0.2% ethyl *m*-aminobenzoate (MS-222) (Sigma). A small incision was made on the abdomen and the ovarian lobes were removed. The follicular cell layer was then removed by digestion with collagenase (2 mg/ml, type IA, Sigma) in OR2 solution (82.5 mM NaCl/2.5 mM KCl/1 mM MgCl₂/5 mM Hepes, pH 7.6 with NaOH) for 2–3 hr. The large oocytes (>1 mm) were then transferred to ND96 solution (96 mM NaCl/2 mM KCl/1.8 mM CaCl₂/1 mM MgCl₂/5 mM Hepes, pH 7.6 with NaOH) and pressure-injected with mRNA. Typically, each oocyte received 40 nl of mRNA solution. Injected oocytes were maintained in ND96 solution supplemented with 2.5 mM sodium pyruvate, 100 units of penicillin per ml, and 100 µg of streptomycin per ml at 18°C. Oocytes were suitable for patch-clamp experiments starting 36 hr after the injection and for up to 7 days.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed.

The incubation medium was changed daily. Immediately before patch-clamp experiments, the oocytes were incubated in a hypertonic "stripping" medium (220 mM *N*-methylglucamine/220 mM aspartic acid/2 mM MgCl_2 /10 mM EGTA, 10 mM Hepes, pH 7.2 with *N*-methylglucamine) for 5–10 min and the vitelline membrane was mechanically removed with fine forceps (25). The results presented are based on the data obtained from 14 patches from myotubes, 12 patches from ShB-injected oocytes, and 10 patches from ShD-injected oocytes.

Electrophysiology and Data Analysis. Unitary currents were recorded using cell-attached, inside-out, and outside-out configurations of the patch-clamp method (26). The data were collected and analyzed essentially as described (27, 28). In excised configurations, the external solution typically contained 140 mM NaCl, 2 mM KCl, 6 mM MgCl_2 , and 10 mM Hepes, adjusted to pH 7.1 with NaOH. The cytoplasmic solution typically contained 140 mM KCl, 2 mM MgCl_2 , 1 mM CaCl_2 , 11 mM EGTA, 10 mM Hepes, and 10 nM free Ca^{2+} , adjusted to pH 7.2 with *N*-methylglucamine. Occasionally, 70 mM KCl was replaced by 70 mM KF. For the cell-attached configuration, the bath solution contained 140 mM KCl, 2 mM MgCl_2 , 11 mM EGTA, and 10 mM Hepes, adjusted to pH 7.1 with *N*-methylglucamine, for myotubes or 98 mM KCl, 3

mM MgCl_2 , 1 mM EGTA, and 10 mM Hepes, adjusted to pH 7.2 with *N*-methylglucamine, for oocytes. Experiments were performed at room temperature (19–22°C).

RESULTS AND DISCUSSION

Whole-cell and single-channel Shaker A-type potassium currents can be recorded from cultured *Drosophila* embryonic myotubes (29, 30). They are eliminated or altered by various Shaker mutations (29, 30). Representative single-channel records are shown in Fig. 1A. Each panel of Fig. 1 shows four current traces elicited by a depolarizing voltage step and an ensemble average generated from a large number of such traces (after each was converted to an idealized record). The upper set of records was recorded during voltage steps to +50 mV; the lower set was recorded during steps to 0 mV. Comparable records obtained from *Xenopus* oocytes after injection of mRNA transcribed from ShB and ShD cDNAs are shown in Fig. 1B and C. Such channels were not seen in uninjected oocytes and were rare in oocytes 1 day after injection. Their density in the membrane increased during the next few days. The general behavior of the native channels and those in the oocyte is similar. In all cases the probability

FIG. 1. Representative openings of A-type channels from an embryonic myotube (A), from a *Xenopus* oocyte injected with ShB mRNA (B), and from a *Xenopus* oocyte injected with ShD mRNA (C). In each panel, a voltage-pulse protocol (Top), four individual records, and an ensemble average showing the time course of the probability of the channel being open (Bottom) are shown. The data shown in the top panels were recorded at +50 mV and those in the bottom panels were recorded at 0 mV following prepulses to -100 or -120 mV. Each of the three patches contained only one active channel. The data in A, B, and C were obtained in the cell-attached, inside-out, and cell-attached configurations, respectively. The pulses were applied every 5 sec (A) or 6 sec (B and C). The data were digitized at 20 kHz. At +50 mV, the data from the myotube (A) were filtered at 2 kHz, those from ShB (B) were filtered at 1.8 kHz, and those from ShD (C) were filtered at 1.2 kHz. At 0 mV, the data were filtered at 2 kHz, 1.2 kHz, and 1 kHz, respectively. Properties of the ShB or ShD channels recorded in the cell-attached configurations did not differ noticeably from those recorded in the inside-out configuration.

of a channel being open is high near the beginning of the pulse and decays to small values by the end of the pulse.

A difference between the native channels and the two Shaker channels expressed in oocytes is their stability in membrane patches. The native channel has a tendency to disappear with time in whole-cell, cell-free, and cell-attached configurations (unpublished data). The ShB and ShD channels, on the other hand, are very stable, even in cell-free patches where recordings as long as 40 min are not uncommon. This behavior of the native channels resembles macroscopic A currents recorded from oocytes injected with ShA or ShC mRNA. These currents tend to run down quickly, sometimes in the first few seconds or minutes, much like the native channels (10, 11). We have had difficulty recording single ShA channels in oocytes, consistent with their being subject to faster rundown.

The native and ShB channels differ in their sensitivity to the potassium channel blocker charybdotoxin. A high-affinity block ($K_d = 4$ nM) of A channels in oocytes injected with Shaker H4 message (identical to ShB message) has been reported (31). We have confirmed the block by charybdotoxin on single ShB channels and the lack of effect of charybdotoxin on native channels in myotubes (data not shown). This indicates that either the structure or the environment of the native channel differs from that of the channel expressed in oocytes. A difference in structure could arise if the native channel protein contained products from other Shaker transcripts or the product of another gene or if different posttranslational processing unmasked a high-affinity site for charybdotoxin in the oocytes.

Although the basic behavior of the three types of channels is similar, there are differences in the time course of the probability of the channel being open as indicated by the ensemble averages (Fig. 2A). Native and ShB averages decay rapidly, whereas ShD averages decay considerably more slowly. This relatively slower decay was evident at other voltages and was highly reproducible among different ShD patches and batches of mRNA. The relative time courses of the ShB and ShD ensemble averages are similar to the previously reported macroscopic currents recorded from ShB and ShD injected oocytes by two-electrode voltage-clamp (12).

Single-channel measurements allow the determination of rate-limiting steps for channel gating and the voltage dependence of transitions between kinetic states that underlie the macroscopic voltage dependence (32, 33). Further, they can provide a quantitative description of which transition rates are altered between the different channel species and how these alterations account for the differences in macroscopic currents. As shown in Fig. 1, openings of Shaker channels occur in short bursts of only a few opening events. Often only one burst occurs during a voltage step, although multiple bursts, separated by closed intervals that are long compared to closed durations during a burst, can occur. Although detailed descriptions of gating require development of kinetic models that can fit all of the macroscopic and single-channel measurements, many important conclusions can be drawn from analysis of single-channel behavior that are relatively independent of a complete kinetic model. The time course of the probability of being open, $P(t)$, can be described by the following convolution integral:

$$P(t) = \int_0^t f(t - \tau)m(\tau)d\tau,$$

where $f(t)$ is the probability density function for the time between the voltage pulse onset and the first opening of the channel (the first latency), and $m(t)$ is the conditional probability of a channel being open at time t given that it first opened at $t = 0$ (34, 35). The voltage dependence of $P(t)$ can result from a voltage dependence in $f(t)$, which has contri-

FIG. 2. (A) Comparison of the time course of the probability of the channel being open at +50 mV among the myotube, ShB, and ShD channels. The traces were scaled so that the peak amplitudes of all three traces are approximately equal. The openings were elicited at +50 mV as in Fig. 1. (B) Voltage dependence of the probability of the ShD channel being open. Openings were elicited at four different voltages following 1-sec prepulses to -100 mV. This patch contained five active channels.

butions from transitions occurring before the first opening, or $m(t)$, which involves transitions occurring after the first opening, or both.

Even though there are quantitative differences in gating between native Shaker channels in cultured myotubes and ShB or ShD channels expressed in oocytes, the voltage dependences of their gating are similar. We have compared $P(t)$, $f(t)$, and $m(t)$ at different voltages to examine the voltage-dependent behavior of the single channels. $P(t)$ was calculated from ensemble averages (Fig. 2). At less positive voltages, the probability of the channel being open increases slowly and decays much more slowly (shown for ShD in Fig. 2B). $f(t)$, the first latency function, was examined in its integral form as a distribution function. The first latency functions at two different voltages are shown in Fig. 3A. $m(t)$ was calculated by synchronizing all records at the time of the first opening and averaging them. These conditional probability functions are shown in Fig. 3B. For both the native channels and the two species of channels in oocytes, the first latency distributions are highly voltage-dependent whereas the behavior after the first opening [$m(t)$] has very little, if any, voltage dependence. In all three cases the voltage dependence of $P(t)$ arises almost exclusively from a voltage dependence of $f(t)$.

The conditional probability function, $m(t)$, has contributions from channels that remain open at time t after first opening and from channels that closed any number of times, reopened at some time earlier than t , and remained open until time t . The relative contribution of these two components to

FIG. 3. (A) Voltage dependence of the first latencies. First latency distributions obtained at two different voltages from myotube (+50 and 0 mV), ShB (+50 and -30 mV), and ShD (+50 and -25 mV) channels are shown. The first latency distribution represents the probability that the channel has opened by the time indicated on the abscissa. (B) Voltage dependence of the conditional probability function, $m(t)$. The $m(t)$ functions at two different voltages obtained from myotube (+50 and 0 mV), ShB (+50 and -30 mV), and ShD (+50 and -25 mV) channels are shown superimposed. The function $m(t)$ represents the probability that the channel was open at time t given that it opened at $t = 0$ (see text). The distributions were obtained by averaging the records with at least one opening after aligning the first opening transition in each depolarizing epoch. Each of the three patches shown (myotube, ShB, and ShD) contained only one active channel.

$m(t)$ can be examined by comparing open duration distributions with $m(t)$. Fig. 4 shows tail distributions of open durations. For native channels, ShB, and ShD, the open duration distributions are not voltage dependent in this range (-20 to +50 mV), and they are adequately fitted by a single exponential function, indicating no need to postulate more than a single kinetically distinguishable open state for all three species. When the open duration distributions are compared to the conditional probabilities, $m(t)$ (the rightmost curve in each graph), it is clear that the three species differ in the contribution of channel reopening to $P(t)$. Native myotube channels tend to open only a single time after a

voltage step, whereas the ShB channels occasionally open additional times. ShD channels, on the other hand, reopen often, with $m(t)$ decaying much more slowly than the open duration distribution. This high reopening frequency is responsible for the much slower decay of $P(t)$ for ShD channels as seen in Fig. 2B, even though the average open durations are usually shorter than those in the native channels. The open durations (mean $\pm$ standard deviation in milliseconds) were as follows: native, 1.7 ± 0.6 ($n = 22$); ShB, 0.92 ± 0.23 ($n = 22$); ShD, 1.2 ± 0.3 ($n = 22$).

The single-channel data are compatible with the following gating scheme. A resting channel must traverse more than

FIG. 4. Comparison of the conditional probability function, $m(t)$, and the open time distributions in myotube, ShB, and ShD channels. In each panel, four functions are shown superimposed: the conditional probability function, $m(t)$, at +50 mV, tail distributions of open times at two different voltages (+50 and 0 mV in myotube, +50 and -30 mV in ShB, and +50 and -25 mV in ShD), and a single exponential tail distribution function fitted to the open time data (solid smooth line). The open time data from myotube, ShB, and ShD channels are fitted with a single exponential tail distribution function with time constants (τ) of 2.0, 0.7, and 1.0 msec, respectively. A tail distribution is the complement of a probability distribution function. It shows the probability that a given duration is greater than time t . Each of the three patches shown (myotube, ShB, and ShD) contained only one active channel. The mean open times of these channels were not markedly affected by the filter frequencies (0.8–2 kHz).

one closed state before opening. The opening pathway is voltage-dependent and accounts for the voltage dependence of the macroscopic kinetics. Transitions out of the open state, including inactivation of open channels, are not voltage-dependent. Inactivated channels can reopen (especially ShD channels), implying that at least one inactivated state is not absorbing. The scheme is the same for native channels as for ShB and ShD channels. Quantitative differences in rate constants can account for their different kinetic behavior. If different Shaker species or other subunits are present in the native channel, they do not result in a qualitatively different mechanism of gating.

This scheme has a striking similarity to the gating of mammalian neuronal sodium channels (34–38). Their voltage dependence comes about from transitions in the opening pathway (first latencies) and inactivation of open channels has very little voltage dependence. It seems that the basic molecular machinery for voltage-dependent opening and non-voltage-dependent inactivation is present in the structure of the Shaker protein, which is one-fourth the size of the sodium channel. The major differences between Shaker channels and sodium channels are the tendencies to burst and return from inactivation (having higher numbers of bursts per trace) in Shaker channels. These vary among the different Shaker species examined here, and probably others as well, based on differences in macroscopic inactivation rates and steady-state current (10–12). The probability of inactivation from resting states is also more pronounced for sodium channels than for Shaker potassium channels (34, 35).

The differences between the native Shaker channels and those expressed in oocytes could reside either in a difference in the channel structure or its environment in the oocyte. This can be addressed by transformation of ShB cDNA into flies deficient for the Shaker locus, where ShB channels can be studied in the native cells. If the differences are not a result of the oocyte expression system, it seems clear that the native channels do not consist entirely of either ShB or ShD polypeptides. The differences in kinetics, stability, and charybdotoxin sensitivity all argue that ShB or ShD channels are at most a small fraction of the native channels in these myotubes.

We thank L. Y. Jan and Y. N. Jan for Shaker cDNA clones and U. J. McMahon for the frogs. This work was supported by National Institutes of Health Grant NS23294 (R.W.A.) and Training Grant NS07158 (W.N.Z.) and by an American Heart Association California Affiliate postdoctoral fellowship (T.H.).

- Thompson, S. H. & Aldrich, R. W. (1978) in *The Cell Surface and Neuronal Function*, eds. Cotman, C. W., Poste, G. & Nicolson, G. L. (Elsevier Biomed./North-Holland, Amsterdam), pp. 49–85.
- Rudy, B. (1988) *Neuroscience* **25**, 729–749.
- Papazian, D. M., Schwartz, T. L., Tempel, B. L., Jan, Y. N. & Jan, L. Y. (1987) *Science* **237**, 749–753.
- Tempel, B. L., Papazian, D. M., Schwarz, T. L., Jan, Y. N. & Jan, L. Y. (1987) *Science* **237**, 770–775.
- Kamb, A., Iverson, L. E. & Tanouye, M. A. (1987) *Cell* **50**, 405–413.
- Baumann, A., Krah-Jentgens, I., Müller, R., Müller-Holtkamp, F., Seidel, R., Kecskemethy, N., Casal, J., Ferrus, A. & Pongs, O. (1987) *EMBO J.* **6**, 3419–3429.
- Schwarz, T. L., Tempel, B. R., Papazian, D. M., Jan, Y. N. & Jan, L. Y. (1988) *Nature (London)* **331**, 137–142.
- Pongs, O., Kecskemethy, N., Müller, R., Krah-Jentgens, I., Baumann, A., Kiltz, H. H., Canal, I., Llamazares, S. & Ferrus, A. (1988) *EMBO J.* **7**, 1087–1096.
- Kamb, A., Tseng-Crank, J. & Tanouye, M. A. (1988) *Neuron* **1**, 421–430.
- Timpe, L. C., Schwarz, T. K., Tempel, B. L., Papazian, D. M., Jan, Y. N. & Jan, L. Y. (1988) *Nature (London)* **331**, 143–145.
- Iverson, L. E., Tanouye, M. A., Davidson, N. & Rudy, B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5723–5727.
- Timpe, L. C., Jan, Y. N. & Jan, L. Y. (1988) *Neuron* **1**, 659–667.
- Noda, M., Ikeda, T., Kayano, T., Suzuki, H., Takeshima, H., Kurasaki, M., Takahashi, H. & Numa, S. (1986) *Nature (London)* **320**, 188–192.
- Noda, M., Shimizu, S., Tanabe, T., Takai, T., Kayano, T., Ikeda, T., Takahashi, H., Nakayama, H., Kanaoka, Y., Minamino, N., Kangawa, K., Matsuo, H., Raftery, M. A., Hirose, T., Naitake, M., Inayama, S., Hayashida, H., Miyata, T. & Numa, S. (1984) *Nature (London)* **312**, 121–127.
- Kayano, T., Noda, M., Flockerzi, V., Takahashi, H. & Numa, S. (1988) *FEBS Lett.* **228**, 187–194.
- Salkoff, L., Butler, A., Wei, A., Scavarda, N., Giffen, K., Ifune, C., Goodman, R. & Mandel, G. (1987) *Science* **237**, 744–749.
- Tanabe, T., Beam, K. G., Powell, J. A. & Numa, S. (1988) *Nature (London)* **336**, 134–139.
- Tanabe, T., Takeshima, H., Mikami, A., Flockerzi, V., Takahashi, H., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1987) *Nature (London)* **328**, 313–318.
- Ellis, S. B., Williams, M. E., Ways, N. R., Brenner, R., Sharp, A. H., Leung, A. T., Campbell, K. P., McKenna, E., Koch, W. J. & Hui, A. (1988) *Science* **241**, 1661–1664.
- Greenblatt, R. E., Blatt, Y. & Montal, M. (1985) *FEBS Lett.* **193**, 125–134.
- Guy, H. R. & Seetharamulu, P. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 508–512.
- Catterall, W. A. (1986) *Trends Neurosci.* **9**, 7–10.
- Seecof, R. L. (1979) *TCA Manual* **5**, 1019–1022.
- White, M. M., Mayne, K. M., Lester, H. A. & Davidson, N. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 4852–4856.
- Methfessel, C., Witzemann, V., Takahashi, T., Mishina, M., Numa, S. & Sakmann, B. (1986) *Pflügers Arch.* **407**, 577–588.
- Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pflügers Arch.* **391**, 85–100.
- Hoshi, T. & Aldrich, R. W. (1988) *J. Gen. Physiol.* **91**, 73–106.
- Hoshi, T. & Aldrich, R. W. (1988) *J. Gen. Physiol.* **91**, 107–131.
- Solc, C. K., Zagotta, W. N. & Aldrich, R. W. (1987) *Science* **236**, 1094–1098.
- Zagotta, W. N., Brainard, M. S. & Aldrich, R. W. (1988) *J. Neurosci.* **8**, 4765–4779.
- MacKinnon, R., Reinhart, P. H. & White, M. M. (1988) *Neuron* **1**, 997–1001.
- Hille, B. (1984) *Ionic Channels of Excitable Membranes* (Sinauer, Sunderland, MA).
- Aldrich, R. W. (1986) *Trends Neurosci.* **2**, 82–86.
- Aldrich, R. W., Corey, D. P. & Stevens, C. F. (1983) *Nature (London)* **306**, 436–441.
- Aldrich, R. W. & Stevens, C. F. (1987) *J. Neurosci.* **7**, 418–431.
- Gonoi, T. & Hille, B. (1987) *J. Gen. Physiol.* **89**, 253–274.
- Kirsch, G. E. & Brown, A. M. (1989) *J. Gen. Physiol.* **93**, 85–99.
- Barres, B. A., Chun, L. L. Y. & Corey, D. P. (1989) *Neuron* **2**, 1375–1388.