Video Article

GABA-activated Single-channel and Tonic Currents in Rat Brain Slices

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Abstract

The GABA$_A$ channels are present in all neurons and are located both at synapses and outside of synapses where they generate phasic and tonic currents, respectively [4,5,6,7] The GABA$_A$ channel is a pentameric GABA-gated chloride channel. The channel subunits are grouped into 8 families (α1-6, β1-3, γ1-3, δ, ε, θ, π and ρ). Two alphas, two betas and one 3rd subunit form the functional channel [8]. By combining studies of sub-type specific GABA-activated single-channel molecules with studies including all populations of GABA$_A$ channels in the neuron it becomes possible to understand the basic mechanism of neuronal inhibition and how it is modulated by pharmacological agents.

We use the patch-clamp technique [9,10] to study the functional properties of the GABA$_A$ channels in alive neurons in hippocampal brain slices and record the single-channel and whole-cell currents. We further examine how the channels are affected by different GABA concentrations, other drugs and intra and extracellular factors. For detailed theoretical and practical description of the patch-clamp method please see The Single-Channel Recordings edited by B Sakman and E Neher [10].

Video Link

The video component of this article can be found at https://www.jove.com/video/2858/

Protocol

1. Preparation of brain slices:

Experiments are done on hippocampal brain slices from postnatal 16 - 22 days old Wistar rats.

1. Animals are sacrificed by decapitation in accordance with local ethical guidelines and approved animal care protocols.
2. Quickly remove the brain with a spatula and cut it along the midline with a surgical blade (# 24). Place each hemisphere on a Whatman filter paper with the cut facing the paper.
3. Put a drop of superglue on the specimen disk and place the hemisphere on top of it with the cut surface facing down. Place the specimen disk in the cutting chamber of the vibratome that is filled with an ice-cold artificial cerebrospinal fluid (ACSF, please see here below 3.1).
4. Set the slice thickness to 400 μM and start cutting the brain. You will need to cut off approximately 2 mm of brain tissue before you reach the hippocampus.
5. The hippocampal slices are put into a glass petri plate filled with ACSF. The plate is placed on a black background (e.g. black paper) to allow easy visualization of the hippocampi. Isolate the hippocampus from the surrounding tissue using sharp surgical blades. Be careful not to push or pull the tissue.
6. Slices are then incubated in the ACSF solution at 37.0 °C for 1h and then stored at room temperature until experiments are done. During the one hour incubation the tissue recovers from the damage imposed by the cutting process.

2. Preparation of pipettes for patching:

1. Pipettes are made from borosilicate glass capillaries.
2. The patch-pipettes are pulled on an automated or manual pipette pullers resulting in pipettes resistance of 2 - 4 MΩ for whole cell recordings or 8 – 20 MΩ for single-channel recordings when filled with the appropriate pipette solution and the bath solution being the ACSF (see below, 3.1).
3. The pipettes we use for single-channel recordings are polished using a microforge where the heat from a melted glass sitting on the platinum wire makes the surface smoother. The same borosilicate glass as is in the patch-pipettes is used to form the glass droplet on the wire. Polishing the pipettes this way in our experience creates pipettes that form more stable and higher resistance seals as compared to pipettes polished by the heat from an uncoated wire or by a polishing step in the automated puller. Final size of the pipettes ranges from 8 to 20 MΩ. Pipettes used in whole-cell recordings are not polished.

3. Solutions used in the experiments:

Specific solutions are used for the single-channel and whole-cell recordings.

1. ACSF contains in mM: 124 NaCl, 26 NaHCO$_3$, 3 KCl, 1.3 MgSO$_4$, 2.5 Na$_2$HPO$_4$, 2.5 CaCl$_2$, 10 glucose with pH 7.2 - 7.4 when bubbled with 95% O$_2$ and 5% CO$_2$. The ACSF is sometimes also supplemented with other nutrients such as e.g. lactate [11].
2. Single-channel recordings: *The bath solution* is either ACSF or in mM: 140 NaCl, 5 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$ and 10 TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) pH 7.25 and other drugs that may be needed for the specific experiments. *The pipette solution* for cell-attached and inside-out patches in mM: 140 Choline Cl, 5 NaCl, 1 KCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 10 TES pH 7.25, 200 µM saclofen (GABAB antagonist) and 0 - 1 µM GABA. For outside-out patches in mM: 140 NaCl or Choline Cl, 0.3 KCl, 2 MgCl$_2$, 0.5 CaCl$_2$, 5 EGTA, 10 TES pH 7.2 - 7.4.
3. Whole-cell voltage-clamp recordings: *The bath solution*: slices are perfused (2 - 3 ml/min) with ACSF now containing also kynurenic acid (3 mM) and other drugs that may be needed for the specific experiments. *The pipette solution* contains in mM: 140 CsCl, 1 CaCl$_2$, 3 EGTA, 0.5 KCl , 1 MgCl$_2$, 2 ATP-Mg, 0.3 GTP-Na, 5 QX-314 bromide, 10 TES pH of 7.25 with CsOH.

4. Forming a Giga Ohm (GΩ) seal for patch-clamp experiments:

The hippocampal brain slices (Fig. 1A) are held at the bottom of the recording chamber by nylon threads on a U-shaped platinum wire. In order to visualize the tissue organization e.g. the CA1 and 3 and the dentate gyrus region in the hippocampal slices or the specific cells, you need a microscope. Most laboratories use an upright microscope with objectives ranging from 10X to 60X thus allowing one to identify regions or specific cells in the tissue. However, for patching slices even a dissection microscope can be used and then you visually identify a region to patch but not a specific cell. This type of patching is termed "blind patching". In both cases one in the end is patching a single cell in a specific area. The success rate is similar for the two methods. Although the cell bodies of the principal cells in the hippocampus are organized into highly structured lamina (e.g. CA3/CA1 stratum pyramidale and identified as bright bands in the hippocampal brain slice) scattered among all subfields and strata of the hippocampus are the cell bodies of inhibitory interneurons that make up about 10% of the total neuronal population [12]. The occasional interneuron or glia that may be patched in the granule cell or pyramidal neuronal lamina can be differentiated from the principal cells by the different electrical properties of these cells (see The Hippocampus Book [13]).

1. GΩ seal formation. During this procedure the glass pipette somehow becomes tightly attached to the cellular membrane and in the different patch configurations (please see below, 4 and 5) we can measure the activity of GABA-activated channels that are in the cell membrane. Put the pipette into the pipette holder and tighten the cap that fastens the pipette. Settings on the Axoclamp amplifier 200B are: V-clamp, Gain 2, Filter 2 kHz, the holding potential is 0 mV and we are doing the experiment via the computer using the PClamp software.
2. Blow into the mouth piece that is attached to the tube that connects to the pipette holder and close the valve between the mouth piece and the tube. This creates positive pressure in your pipette. The positive pressure in the pipette has two functions; first, a stream will come out of the pipette and once the pipette enters the bath solution it will help keeping the pipette tip clean. Second, applying a gentle suction to the cell membrane through the patch-pipette is a vital part of forming a high quality, high-resistance seal. The seal often can be simply formed by opening the valve that controls the pipette pressure. Because of this, make sure there are no air leaks. You can easily test for leaks by taking the holder with the pipette, dip it into a beaker filled with water and blow into the mouth piece. If you see any bubbles escape then that part is not tight enough.
3. Lower your pipette into the bath solution. On the computer screen where you measure the pipette resistance you will see a square pulse generated by a 5 mV pulse train and a number that tells you the resistance of your pipette. Smaller number represents larger pipette tip openings, larger pipettes. Adjust the pipette offset to 0.
4. Sealing to the cell membrane. Identify the region or the cell you are going to patch, that is, form a seal on. Within the brain slice one can identify the dentate gyrus granule or the CA1 and CA3 pyramidal cell body regions of the hippocampus as bright bands (see Fig. 1A) using the 10X magnification on the microscope or we can identify the individual surface pyramidal neurons using the 60X magnification.
   1. Bring your pipette into focus so that you see both your region / cell and the pipette when you look through the microscope eye-pieces.
   2. Place the pipette above the middle of the region / cell using the coarse-movement manipulator settings and lower the pipette until it is just above but not touching the tissue.
   3. Very gently lower your pipette onto the middle of your region / cell using the fine-movement settings on your manipulator and at the same time watch the computer screen and keep track of your pipette resistance. Keep moving your pipette down until you see the resistance of the pipette becoming larger (higher number).
   4. Now, gently release the positive pressure in the pipette by opening the valve. This amounts to a gentle suction and often a giga-seal (high resistance seal, GΩ) forms automatically.If not, then apply suction to the mouth piece where you previously applied positive pressure and the *Giga-Seal* will form.
   5. You have now obtained the so-called *Cell-Attached* patch-clamp configuration (see Fig. 1B). Adjust the fast and slow capacitance compensation to cancel the transients coming from the pipette and the pipette holder. On the computer screen you will now see a flat current trace. The higher the GΩ seal value is, the better signal-to-noise ratio you will have in the single-channel recordings.

It can sometimes be advantageous to patch within the hippocampal slice. In some instances the cells can be healthier when not on the surface as they are more protected. Also, in case one is studying the effects of the extracellular environment on cellular functions, such as effect of extracellular GABA concentrations on tonic current generation in neurons, patching within the tissue can be an advantage. The patching procedure is the same as described above apart from that one does not release the positive pressure in the pipette until the pipette tip is within the slice.

5. Establishing the whole-cell configuration and recording whole-cell currents:

1. In the cell-attached configuration, change the pipette potential to -60 mV or close to the resting membrane potential of your cell.

2. Apply suction to the pipette via the tube that is connected to the pipette holder and at the same time watch the computer screen to note the change in resistance and the capacitance transients that appear once you have broken into the cell. When this happens you are in the so-called *Whole-Cell configuration* or Whole-Cell mode (Fig. 1B).
    The cell membrane resistance varies from one cell-type to another and depends on the conductance of the channels in the membrane and how many are open. The cell membrane capacitance varies with the size of the cell. Suction should initially be gently but if it is difficult to break into the cell more force can be applied. Pulses of suction can also be used.
3. Sometimes a compensation for resistance that develops between the pipette and the cell is required. This is called series or access resistance (Rs or Ra) compensation and can be done either via the software or manually using a control knob on the amplifier. In our experiments we do not compensate for Ra but experiments are rejected if it varies by more than 25% from the initial access resistance.
4. GABA-activated tonic currents recorded in the whole-cell mode.

In the whole-cell configuration in hippocampal CA1 pyramidal neurons we set the holding potential at - 60 mV and wait for 5 - 10 minutes allowing the pipette solution to equilibrate with the cell interior. During this time the recording becomes stable. During the experiment we keep track of the value of the series resistance.

Experiments are done in voltage-clamp and you measure the current through a population of channels in the whole-cell membrane. One can differentiate between GABA-activated synaptic and extrasynaptic currents by the phasic and tonic nature of the currents. The antagonist SR95531 inhibits all GABA$_A$ currents. The decrease in the holding current indicates the level of the GABA-activated tonic current in the neuron.

As we are interested in the tonic GABA-activated current that is dependent on the extracellular, ambient GABA in the slice we normally do not patch the cells on the surface but rather, the cells within the slice. Agonists and antagonists that we use to modulate the GABA$_A$ channels are applied in the bath solution and do reach the cells within the slice. The rate of the solution flow in the experimental chamber is 2 - 3 ml / min.

6. Recording single-channel currents:

Experiments are done in the cell-attached, the inside-out or the outside-out patch-clamp configuration (please see here below) and we record currents through single-channel molecules, the single-channel currents. Settings on Axoclamp 200B, V-clamp, gain 500, filter 2 or 5 kHz. With the PClamp software we control the pipette potential, set the data sampling rate at no less than 100 μs and record the currents. In order to minimize noise in single-channel recordings the volume of the bath solution is kept low so that it just covers the slice.

1. GABA-activated channels in the Cell-Attached configuration. When you have obtained a GΩ seal on the membrane you are in the cell-attached mode and you can record from the channels that are in the membrane patch enclosed by the pipette (see Fig. 1B). As GABA does not cross the cell membrane, GABA is put into the pipette solution to activate the channels in the patch. Extrasynaptic GABA-activated channels activate with a latency of up to few minutes. The patch potential is = - pipette potential i.e. when in PClamp you set the potential at - 40 mV, it is recorded by the membrane as depolarized 40 mV.
    The advantage of recording in the cell-attached mode is that the channel is in its native environment and is in connection with the normal intracellular proteins and factors. The disadvantage is that you do not know at exactly what potential you are recording as you do not know the absolute resting membrane potential and the potential across the patch is determined both by the pipette potential and the resting membrane potential of the cell.
2. GABA-activated channels in the Inside-Out configuration. When in the cell-attached mode gently lift up your pipette and move it to the side and away from the slice by using the fine movements of the micromanipulators. You have now an inside-out patch and can record in the Inside-Out mode.
    You record from the patch of membrane that is enclosed by your pipette (see Fig. 1B). As in the cell-attached mode you have GABA in the pipette solution to activate the channels. But, drugs that cross the lipid bilayer such as benzodiazepines, barbiturates, propofol etc. can be applied in the bath solution as they will be able to cross into the pipette and modulate the channels [14]. The patch potential = - pipette potential and since there is no resting membrane potential overlaid as in the cell-attached mode, the patch potential is equal to the -pipette potential e.g. if you set the holding potential in PClamp to -40 mV then the patch potential is exactly 40 mV.
    The advantage of the inside-out configuration is that one can apply drugs or enzymes to the internal membrane surface and examine if it affects channel properties. The force of making the inside-out patch does not apply to the membrane environment of the channel making the formation of this ripped-off patch configuration potentially more gentle for the channel complex than when the outside-out patch is formed (please see below).
3. GABA-activated channels in the Outside-Out configuration. When in the whole-cell mode gently lift up or draw back the pipette using the fine movements of your micromanipulator. At the same time watch the window on your computer that displays the patch properties. As you draw the pipette away from the cell you will see the capacitance transients disappear and you will again have a GΩ seal. You have now formed an outside-out patch and can record in the Outside-Out mode.
    Somehow the cell membrane has sealed over the tip opening of the pipette, the inside of the membrane faces the pipette interior and the original outside of the membrane faces the recording chamber (Fig. 1B). GABA and other drugs to be examined are now dissolved in the bath solution. The patch potential = pipette potential just like in the whole-cell mode e.g. if you set the holding potential in PClamp to 40 mV then the patch potential is exactly 40 mV.
    The advantages of the outside-out mode is that you know precisely what your membrane potential is and as the membrane original outside faces the experimental chamber, drugs to be used can be dissolved in the bath solution and easily applied to and washed off the channels. The disadvantages are that you no longer may have attached to the channel intracellular and even transmembrane proteins and factors that may be needed for full channel function as the membrane had to seal-over and some molecules may have been lost or the local membrane environment distorted in the process. But, together the different patch configurations allow dissection of channel properties at the molecular level that is not possible with any other method today.

7. Analyzing the results:

The single-channel and whole-cell currents are analyzed with the PClamp software. For analyzes of synaptic events we use Synaptosoft (GA, USA) or AXOGraph (Sydney, Australia).

8. Representative results:

Fig. 2A shows single-channel currents activated by 20 nM GABA concentration in a cell-attached patch in dentate gyrus granule neuron. The latency of activation of the channel's maximal conductance was 2 minutes and 38 seconds and the maximal conductance was 44 pS [15]. The patch was depolarized by 40 mV. Fig. 2B shows examples of whole-cell voltage-clamp current records from rat hippocampal neurons identifiying tonic and phasic currents. The GABAA antagonist SR95531 inhibits both the extrasynaptic- (tonic) and the synaptic- generated (phasic) GABA-activated currents. We applied the GABAA antagonist SR95531 (100 µM) to (Ba) dentate gyrus neuron and (Bb) insulin treated CA1 pyramidal neuron revealing the tonic current by the shift in the baseline current induced by SR95531 blocking the extrasynaptic channels (Ba,b) and the disappearance of synaptic currents when SR95531 blocks the synaptic channels (Bb). Under basal condition CA1 pyramidal neurons do not have tonic GABA-activated currents [6] but when the neurons are exposed to physiological concentrations of insulin [4] GABA-activated tonic currents develop.
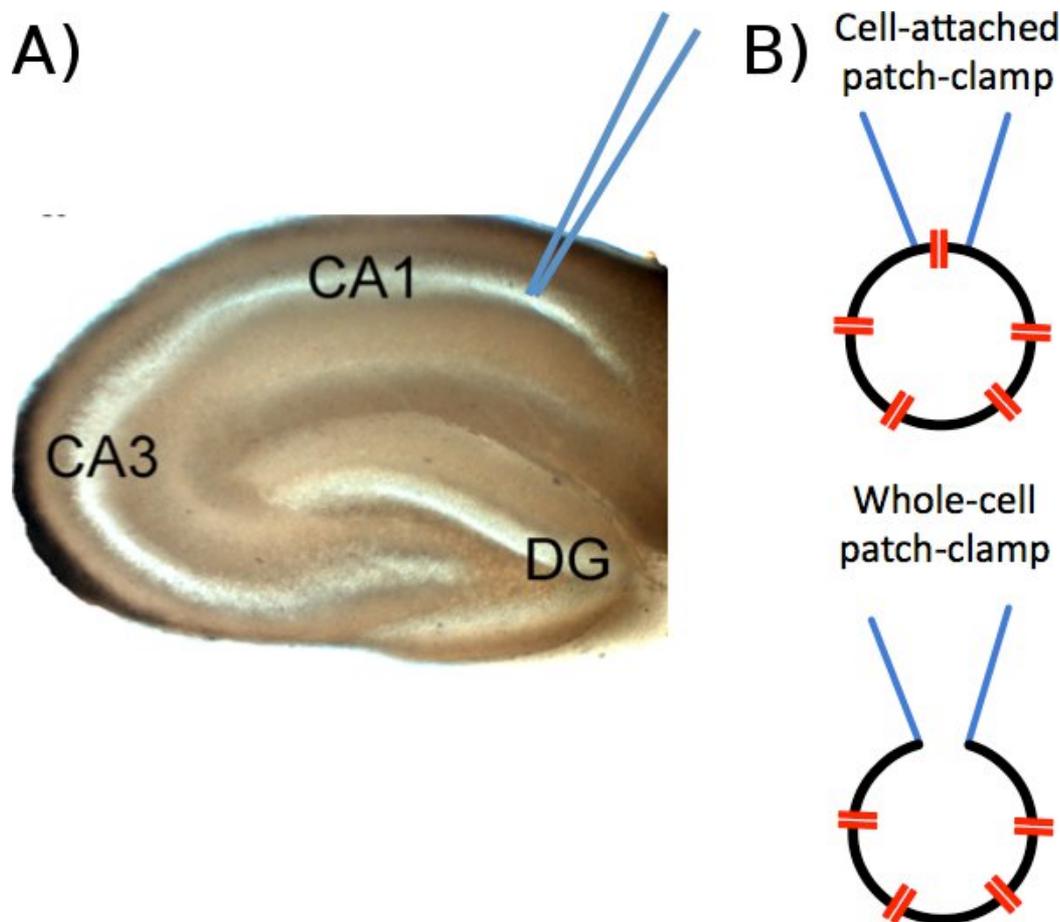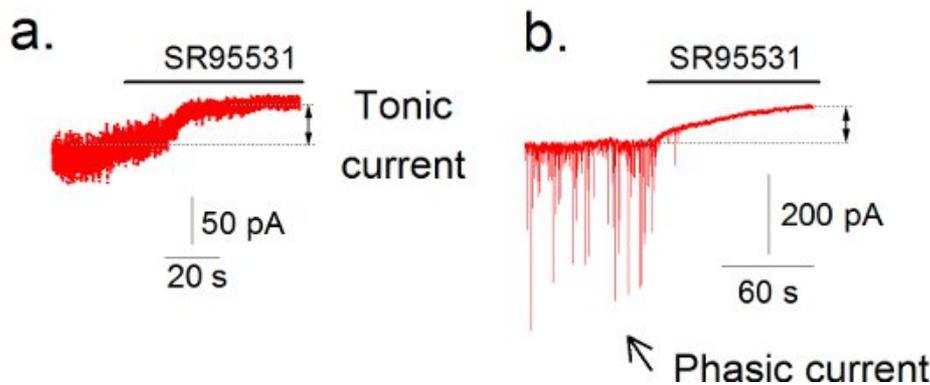

Figure 1A. Rat hippocampal brain slice, regions identified are CA1 and CA3 pyramidal cell layers and the dentate gyrus (DG) granule cell layer. **B.** Schematic patch-clamp configurations.

A. Single-channel currents
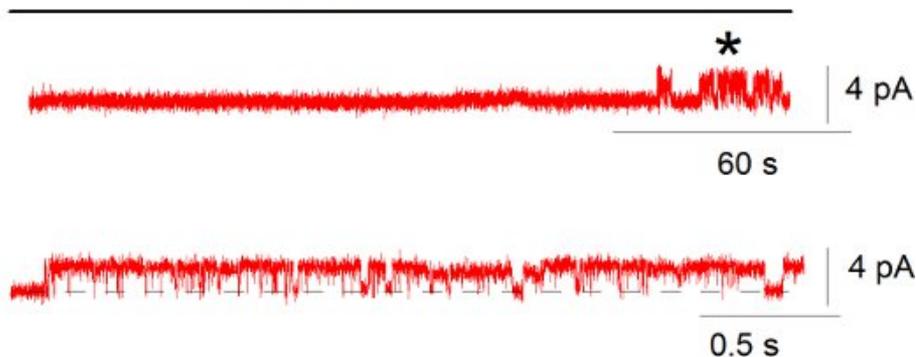
B. Whole-cell currents

Figure 2A. Single-channel currents activated by 20 nM GABA in a cell-attached patch on dentate gyrus granule cell in a rat hippocampal brain slice. Holding potential was depolarized 40 mV (pipette potential = -40 mV). The star (*, 1st current trace) identifies from where the currents shown on the faster time-scale (2nd current trace) was taken from. **B.** Whole-cell currents recorded in rat hippocampal brain slice in **a.** dentate gyrus granule cell and **b.** CA1 pyramidal neuron in voltage-clamp mode at the holding potential of -60 mV. For the CA1 experiments, slices were incubated in 1 nM insulin for 2 h at room temperature before currents were recorded.

Discussion

The whole-cell current represents the ensemble of currents generated by active channels in the cell. The specific pharmacology and chloride permeability of the GABAA channels makes it possible to identify currents associated with these channels. The transient nature of activation of synaptic channels and the tonic activation of the extrasynaptic channels allows easy differentiation between these two populations of GABAA channels expressed in a neuron. However, only with single-channel recordings can one study the kinetic properties of the channel molecules that generate the tonic currents. Unlike synaptic channels that activate within 100 μs, the tonic GABAA channels are activated with a latency in the range of minutes from the time GABA is applied [4,7,15,16,17,18]. This delayed activation of the channels results in that many channel properties cannot be studied using whole-cell recordings. At the single-channel level the different sub-populations of GABAA channels can be identified based on varying functional and pharmacological properties. Thus, by taking an advantage of what the different patch-clamp configurations have to offer, it is possible to study in detail the molecular function and pharmacology of the GABA-activated channels that generate the tonic and synaptic currents in neurons.

Disclosures

No conflicts of interest declared.

Acknowledgements

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