Excitability tuning of axons in the central nervous system

Shunsuke Ohura1 · Haruyuki Kamiya1

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Abstract The axon is a long neuronal process that originates from the soma and extends towards the presynaptic terminals. The pioneering studies on the squid giant axon or the spinal cord motoneuron established that the axon conducts action potentials faithfully to the presynaptic terminals with self-regenerative processes of membrane excitation. Recent studies challenged the notion that the fundamental understandings obtained from the study of squid giant axons are readily applicable to the axons in the mammalian central nervous system (CNS). These studies revealed that the functional and structural properties of the CNS axons are much more variable than previously thought. In this review article, we summarize the recent understandings of axon physiology in the mammalian CNS due to progress in the subcellular recording techniques which allow direct recordings from the axonal membranes, with emphasis on the hippocampal mossy fibers as a representative en passant axons typical for cortical axons.

Keywords Axon · Initial segment · En passant axons · Mossy fiber · Propagation failure · Subcellular recording

Morphology of axon

Axons are generally composed of three compartments, i.e., the axon hillock, the shaft, and the presynaptic terminal [1] (Fig. 1). The axon hillock is the compartment of neuron that connects the soma and the axon. The adjacent initial part of the axon, which is called the axon initial segment (AIS), usually generates action potentials in the physiological conditions [2, 3]. The axon shaft typically forms varicosities of presynaptic terminals with a bouton-like appearance along their course. These boutons are distributed as a “pearl-chain structure”, and often called en passant axons [4]. For instance, hippocampal mossy fibers have approximately ten boutons per each axon on average [4]. Each bouton of en passant axons contains numerous synaptic vesicles and multiple release sites, and synaptically connected with a number of postsynaptic neurons. Although the en passant axons enable nearly simultaneous output to multiple target neurons, they are also known as a risk factor of propagation failure of axonal spikes by “impedance mismatch” of thin axon shafts and large boutons. It is intriguing that hippocampal mossy fiber boutons express a high density of Na+ channels, which ensure the high fidelity of spike propagation and amplify the amplitude of the presynaptic action potentials for supporting the efficacy of transmitter releases [5].

The morphology of axons in the central nervous system (CNS) is classified in terms of myelination and arborization. Myelinated axons are covered by a myelin sheath composed of oligodendrocytes, and are well suited for saltatory conduction between the nodes of Ranvier, which speed up the velocity of action potential propagation along the axons [6, 7]. Axons of the layer 5 neocortical pyramidal cells, the cerebellar Purkinje cells, and the spinal cord motoneurons are representative myelinated axons which have been studied most extensively using electrophysiological experiments [1]. In the layer 5 neocortical pyramidal neurons, the conduction velocity was estimated to be 2.9 m/s [8], while it was 70–80 m/s in the motoneurons [9]. On the other hand, unmyelinated axons were not covered...
by a myelin sheath, which makes the propagation velocity much slower than in myelinated axons. Hippocampal mossy fiber, Schaffer collaterals, and cerebellum parallel fiber are all representative unmyelinated axons that are well characterized functionally with electrophysiological experiments [1]. The conduction velocity of Schaffer collateral and hippocampal mossy fibers is estimated to be 0.25 m/s [10, 11].

In addition to myelination, the pattern of axon arborization or branching may affect the information flow and functional properties of the axon. The branching pattern of axons varies between the cell types. Hippocampal CA3 pyramidal cells had at least 100–200 branch points [12]. Moreover, hippocampal basket cells, an inhibitory interneuron, have much more axonal arborization for the synchronization of a large population of the targeted cells during network activity and oscillation [13, 14]. Axon branching is also known as a risk factor of propagation failure because of the “impedance mismatch” of parent and daughter axons [15].

**Subcellular recordings from the axon in the CNS**

The first quantitative description of axonal membrane excitability was achieved through electrophysiological experiments in the squid giant axon, which could be easily impaled with glass microelectrodes because of its exceptionally large diameter (~400 μm) [16]. Density and gating of basic ionic channels, i.e., Na⁺, K⁺, and leak conductances, were examined rigorously with the voltage-clamp method, and the theoretical model with the equation describing the dynamics of axonal membrane potential successfully predicted the action potentials precisely similar to those obtained experimentally. It should be noted that the Hodgkin Huxley model has been often used to predict membrane potential changes and network dynamics of CNS in many recent modeling studies.

Local control as well as activity-dependent tuning of axonal excitability need to be investigated in order to elucidate the mechanisms underlying information processing and circuit functions in the brain. However, the direct electrophysiological recordings from an axonal membrane in the CNS were almost impossible because of the small size of diameter of axon shafts (0.2–1 μm in diameter) and the complex arborization. Recent attempts to record directly from axons by visualizing the axon shafts and the terminals with the aid of an infrared differential interference microscope (with or without fluorescent labelings) overcame the limitation of recordings from these small structures. The first subcellular recording of a mammalian CNS neuron was obtained from the dendrite and the axon of neurocritical pyramidal cell using whole-cell current clamp recordings [17]. Using this approach, they uncovered the backpropagation of axonal action potentials into the dendrites, which is important for dendritic integration and plasticity.

Thereafter, the efforts for recording from axon terminals (Fig. 2a) were directed to the auditory relay synapse in the brainstem, so-called the calyx of Held [18]. This exceptionally large axon terminal was well suited for whole-cell recording from the axon terminals, and therefore was used not only for studying the ionic basis of excitability but also for elucidating the molecular mechanisms of neuronal transmitter release. The calyx of Held is the best-studied axon terminals in the CNS, and is the current leading model of the studies in the presynaptic mechanisms. In addition, large axon terminals of hippocampal mossy fibers were also studied extensively by subcellular recordings.

![Fig. 1 Schematic drawing of axon in the mammalian CNS. Axons often make multiple varicosities (or boutons) and multiple branching. The most proximal part of an axon, adjacent to axon hillock, is called the axon initial segment (AIS), and generates action potential. It propagates forward to the distal axons and the boutons (presynaptic terminals), and also backpropagates to the somato-dendritic domain.](image1)

![Fig. 2 Configurations of subcellular recordings from axons. a Recording from axon terminals/boutons. b Recording from axonal bleb, a spherical structure formed at the cut end of axons by tissue slicing procedures. c Recording from the intact axon shaft.](image2)
[19, 20] with whole-cell configuration. Hippocampal mossy fibers form en passant synapses with multiple postsynaptic neurons, and display unique presynaptic forms of synaptic plasticity. It had been shown that a high density of voltage-dependent Na\(^+\) conductance at the mossy fiber boutons ensured the propagation of action potentials over multiple boutons on en passant axons [5].

Another approach to assess the ionic channels in the axon is to record from axonal blebs (Fig. 2b). While making brain slices, the cut end of the axon on the surface of slice gets sealed to form spherical structures of 3–6 μm in diameter, which are called “axonal bleb” and are well suited for patch-clamp recording. This method revealed distribution of ionic channels in axon shafts which are usually too thin to access with the recording pipette. In previous studies, the axonal bleb recording was applied to the axons of the layer 5 neocortical pyramidal cells [8, 21], the hippocampal CA3 pyramidal cells [22, 23], and the hippocampal granule cells [24]. By systematic efforts to directly record ionic channels along axon, axonal bleb recording approach revealed important aspects for understanding axon physiology including the distribution of maximal conductance and difference in gating kinetics of voltage-dependent Na\(^+\) channels, as well as the exact site of spike initiation in the AIS.

Although the recording from axonal blebs supplies direct information on ionic conductance of the axon shafts, it may not reflect the properties of intact axonal membrane in the physiological conditions. A previous study demonstrated that the resealed bleb could cause an increase in the length constant of the axonal cable, which had to be considered that the passive properties of the axonal membrane can be changed in the axonal blebs [21]. To overcome this issue, recent studies have successfully obtained the direct recording from the intact axonal shaft (Fig. 2c). Targeted cell-attached recordings from axons of hippocampal pyramidal cells in culture, with the aid of a glass pipette coated with Alexa Fluor-conjugated albumin, revealed analogue modulation of action potentials during conduction along the axon [25]. Recently, direct whole-cell recordings from the axon shaft were performed on the basket cells in the hippocampus, and revealed that the high density of Na\(^+\) channels at the distal axons enabled reliable spike propagation even with extensive branching for the synchronous network activity of neuronal ensembles [14]. In contrast, whole-cell recordings from the axon shaft and the terminal of cerebellar Purkinje cells in culture revealed that the low excitability of the distal axons due to the low density of Na\(^+\) channels may cause activity-dependent conduction failure at the distal axons leading to synaptic depression [26]. As is obvious in these previous studies, advances in the recording techniques can provide new insights on the understandings of axon properties and functions in the CNS.

**Distribution of Na\(^+\) channels**

The subcellular recordings from axons have demonstrated the distribution of ionic conductances along axons, especially of voltage-dependent Na\(^+\) channels, which are most important for initiation of action potentials and their propagation. Direct recording from axons also revealed the differences in the properties of ionic channels, such as gating kinetics as well as voltage-dependency, in the axon from those in the soma. In general, the expression of Na\(^+\) channels is much higher in the axon, especially at the AIS, to support reliable spike initiation at the AIS and safe spike propagation along the axon.

In myelinated axons of neocortical pyramidal neurons, it was demonstrated that the density of voltage-gated Na\(^+\) (Na\(_{v}\)) channels in the AIS were 40–50 times higher than that of the soma [27, 28]. In unmyelinated axons of hippocampal mossy fibers, the density of Na\(_{v}\) channels in the AIS was estimated to be fivefold higher than that in the soma [29]. These electrophysiological data are consistent with the findings of morphological study with immunohistochemistry. In hippocampal mossy fibers, the average staining of the Pan Na\(_{v}\) immunoreactivity, which detects the expression levels of Na\(_{v}\) channels, was found to exist about several folds higher in AIS than in the soma and peak at proximal axon at 18 μm from the soma [11]. The spatial distribution of immunoreactivity of AIS marker ankyrin G was also the same propensity as the Pan Na\(_{v}\) signal. These data indicate that the density of Na\(_{v}\) is highest at AIS along somato-axonal axis of unmyelinated mossy fibers.

Direct electrophysiological recording from AIS in combination with immunohistochemistry revealed a refined distribution of sodium channel subtypes within AIS. In layer 5 neocortical pyramidal neurons, low threshold type Na\(_{v}\)1.6 was distributed at the distal portion of an AIS, which promoted action potential initiation at the distal portion of AIS, whereas high threshold type Na\(_{v}\)1.2 was distributed at the proximal axon and enabled backpropagation of action potentials to the somato-dendritic compartment [28].

The Na\(_{v}\) channel properties in the presynaptic terminals have also been investigated. A previous study showed that the density of Na\(_{v}\) channels was very low at the presynaptic terminal of calyx of Held [30]. Therefore, the action potential invasion into the presynaptic terminals is supposed to be a passive process. In contrast, the direct recording of the hippocampal mossy fiber terminals revealed a high density of Na\(^+\) conductance of around 490 pS/μm\(^2\) [5]. Presynaptic Na\(^+\) channels have been shown to
amplify the presynaptic action potentials and boost the presynaptic Ca$^{2+}$ influx.

The density of Na$_v$ channel in the axon shaft has been also examined by recording from axonal bleb. In hippocampal mossy fibers, Na$_v$ channel density was highest in the proximal parts of the axon and gradually decreased towards the distal part of the axon [29]. The Na$_v$ channel density in the distal axon was estimated to be 400 pS/µm$^2$, which is very close to that in mossy fiber bouton [5, 29].

### Distribution of K$^+$ channels

Voltage-dependent as well as Ca$^{2+}$-dependent K$^+$ channels contribute to the repolarizing phase of action potentials in the central nervous system. Molecular diversity as well as use-dependent or neuromodulator-dependent regulation of K$^+$ channels is well suited for the analogue tuning of the excitability of axon. Although the Hodgkin–Huxley-type model assumes non-inactivating (delayed rectifier) voltage-dependent K$^+$ channels in squid giant axons, it has to be mentioned that the voltage-dependent K$^+$ channels in the CNS axons include inactivating-type K$^+$ channels such as K$_v$1 or K$_v$3.

In most parts of the CNS axon, Na$_v$ channels are exclusively composed of three isoforms: Na$_v$1.1, Na$_v$1.2, and Na$_v$1.6. For example, Na$_v$1.2 is homogeneously distributed in unmyelinated fibers and supports the action potential propagation [15]. In contrast, there are a wide variety of subtypes of voltage-gated K$^+$ (K$_v$) channels distributed in the CNS axon. K$_v$ channels were classically thought to accelerate the repolarizing phase of action potentials [1]. Thanks to advances in subcellular recordings, additional functions of K$_v$ channels have been explored. For example, K$_v$1, K$_v$3, and BK$_{Ca}$ (large-conductance calcium- and voltage-activated potassium channels) were located on the hippocampal mossy fiber boutons [19, 31], which differently contributed to presynaptic action potential repolarization. During the repetitive stimulation, action potential waveforms were broadened in the axon terminal but not in the soma [19], which could be caused by the accumulation of K$_v$1 channel inactivation. This activity-dependent, short-term broadening of the action potentials contributes to increases in the presynaptic Ca$^{2+}$ influx and enhances transmitter release. Importantly, axonal action potentials can be amplified or broadened during the repetitive action potentials, and therefore are susceptible to analogue modulation during propagation along the axons, in contrast to the prevailing notion of digital signal processing in the axon.

In cerebellar stellate cells, K$_v$1 channels determined action potential repolarization at the AIS, while K$_v$3 channels contributed to the spike repolarization at the presynaptic boutons [32]. Localized K$_v$1 expression at the AIS limits use-dependent broadening of action potentials to the proximal axons. On the other hand, K$_v$3 at the boutons uncouples use-dependent modification of action potential at the release sites. K$_v$7/KCNQ channels were found in the AIS and determined the excitability of pyramidal CA1 cell axons [33]. In layer 5 neocortical pyramidal neurons, K$_v$7 channels and Na$_v$ were colocalized at AIS and nodes of Ranvier [34]. Interestingly, these axonal K$_v$7 channels were minimally recruited during an action potential, while nodal K$_v$7 channels were found to increase Na$_v$ channel availability and action potential amplitude by stabilizing the resting membrane potential [35]. In hippocampal granule cell axons, the activity of axonal K$_v$7 channels were attenuated by decreasing axonal intracellular Ca$^{2+}$ levels, which decreased the action potential threshold [36].

### Unique properties of axonal Na$^+$ channels

The classical description of dynamic properties of gating of ion channels is based on the Hodgkin and Huxley model (HH model) composed of the three basic ionic channels, i.e., Na$^+$, K$^+$, and leak conductance [16]. This model was based on the experiments performed on the squid giant axons more than 60 years ago, and is still useful to simulate the neuronal excitation and network activity in recent numerical simulation studies. However, recent direct recordings from mammalian CNS axons revealed more complex gating and variable kinetics of ion channels than those in the squid giant axons.

Depending on the neuron types, the kinetic model of gating of Na$^+$ and K$^+$ channels was modified from those in the original HH model to better fit the experimental data. Direct recordings from hippocampal mossy fibers revealed the slightly faster gating of axonal Na$_v$ channels [5]. In addition, the gating model of axonal Na$_v$ channels on the mossy fibers assumes more complex processes for activation. Although the classical HH model assuming three identical rate constants [16] does not accurately describe the behavior of axonal Na$_v$ channels, the six-state [37] or the eight-state gating models [29] have been proposed to fit the experimental data.

It was noted that the properties of axonal ionic channels are different from those in the soma. In the layer 5 neocortical pyramidal neurons or hippocampal mossy fibers, it has been reported that Na$_v$ channels of the proximal axon activate and inactive faster than somatic Na$_v$ channels [21, 28, 29, 38]. These kinetic differences contributed to optimize the efficacy of energy consumption of the axonal membrane during action potential generation. The action potential is generated by inward current through Na$_v$ channels and subsequent outward current through K$_v$. [Springer]
channels. Since the Na\(^+\) influx and K\(^+\) efflux counteract for membrane polarization, the time overlap between inward Na\(_v\) channel currents and outward K\(_v\) channel currents during the action potentials consume more energy for settling the ionic imbalance caused by generation of action potentials by activating ionic pumps for Na\(^+\) and K\(^+\) movements. A previous study demonstrated that influx of fourfold of Na\(^+\) ions of the theoretical minimum (without overlap of counterflow of K\(^+\) efflux) is required to generate action potentials in squid giant axon [16]. In hippocampal mossy fibers, however, a fast onset and fast decay of Na\(^+\) current and a delayed onset of K\(^+\) current could minimize the overlap of their respective ion fluxes, which results in reducing energy demand for generating action potentials to only 1.3 times of the theoretical minimum [39]. Therefore, axonal Na\(^+\) and K\(^+\) channels seem to be optimized in terms of efficacy of energy consumption of the neuron for activating ionic pumps in the axonal membranes to balance ionic environment during neuronal activity.

**Site of spike initiation**

The pioneering study in the spinal cord motoneuron demonstrated that action potentials were generated in the axon initial segments (AIS) and propagated orthodromically to the presynaptic terminals and also invaded into somatodendritic compartments [21]. On the other hand, under specific conditions, action potentials can be initiated in the dendrites of cortical neurons [40], hippocampal neurons [41], or cerebellar Purkinje cells [42]. The detection of spike initiation sites under physiological conditions was conducted using simultaneous axon-soma recordings in the layer 5 neocortical pyramidal neurons [3]. Based on the comparison of the spike latencies between the axon and the somatodendritic compartments, it had been found that the onset of action potentials in the proximal axon was always faster than that in the soma. This data supported the idea that the proximal axon is the spike initiation site under physiological conditions. Further studies with systematic changes in the recording sites revealed the spike initiation sites of the axon in more detail. For example, the initiation sites of unmyelinated axons are estimated to be located between 20 and 40 \(\mu\)m from the soma [14, 29, 43] and those of myelinated axons were estimated to be between 15 and 40 \(\mu\)m from the soma [3, 9, 44, 45]. Based on these studies, it has been demonstrated that action potentials are generated in the proximal axon (AIS) and propagate orthodromically towards the axon terminals.

Although the previous study in the spinal cord motoneuron predicted that the voltage threshold was 10–20 mV lower in AIS than in the soma [2], there was little experimental evidence in mammalian neurons. Direct dual current clamp recordings from the AIS and the soma of layer 5 neocortical pyramidal neurons showed that the current threshold to elicit an action potential was lower in the AIS [46], as expected from the high density of Na\(_v\) channels in the AIS. Interestingly, the voltage threshold defined as the membrane potential was higher in the AIS than in the soma. This surprising observation was interpreted as follows. Highly abundant expression of the voltage-dependent Na\(^+\) channels in AIS not only initiates action potentials at the AIS but also drives a slow subthreshold depolarization at the AIS prior to the fast action potential. Since this subthreshold depolarization does not propagate to the soma, somatic action potentials showed apparently lower voltage threshold than those recorded at the AIS [46].

In some specific conditions, the spikes are exceptionally generated in distal axons and propagate antidromically towards the somatodendritic compartments. These ectopic spikes were initially observed under pathological conditions of hyperexcitability [47], but recent studies demonstrated that these ectopic spikes can occur even in physiological conditions. In hippocampal CA1 interneurons, repeated and prolonged somatic current injections triggered persistent firings, which outlast stimulation period and continued after stopping the current injection, and were suggested to generate in the distal axon. This unique plasticity of axonal excitability was called persistent firing or retroaxonal barrage firing [48]. Furthermore, ectopic spikes were also shown to be generated in the CA3 pyramidal cell during gamma-range frequency oscillation [49] or in the CA1 pyramidal cell during sharp wave ripple [50]. Although the exact mechanisms of generation of ectopic action potentials remain to be determined, it was suggested that gap junction may contribute to this persisting change in axonal excitability [51, 52]. The possible function of ectopic spikes is maintenance of fast neuronal network activity [13, 14]. In addition, the ectopic spikes in neuroglialform (NG) interneurons of the hippocampus monitored excitability of their neighboring cells, providing a distributed inhibition that can outlast the original stimulus to prevent hyperexcitability [53]. The underlying mechanisms and functional significance of ectopic spike generated at distal axons, however, still remains largely unknown.

**Fidelity of spike propagation**

Under physiological conditions, the axons of mammalian neurons carry action potentials with high fidelity. However, in some specific conditions, action potentials generated in the AIS cannot propagate faithfully towards the presynaptic terminals, and are called propagation failures. Both
geometrical factors (such as arbolization and varicosity formation) as well as channel properties (inactivation of Na, channels) are important for determining the propagation failure.

The axon branching and the arborization of neurons are risk factors of the propagation failure [54–56]. It was also noted that propagation failures occurred when action potentials enter into the axon terminals or en passant boutons [57, 58]. It was also pointed out that failure may also occur when antidromic spikes invade the soma [59]. The propagation failure happens due to “impedance mismatch”, which occurs when an action potential enters a space with a different diameter [15].

The repetitive stimulation of axons caused the frequency-dependent propagation failure. For example, axonal spikes occasionally failed when the CA3 pyramidal neuron fired at 30–40 Hz [60]. In cerebellar Purkinje cells, axonal spikes failed when firing exceeded 250 Hz [61]. On the other hand, mossy fibers in the cerebellum had been shown to have higher fidelity of spike propagation up to 1.6 kHz [62]. The spike propagation may fail, at least in part, as a result of accumulation of K ions around the axon and prolonged depolarization of the membrane potentials which cause steady-state inactivation of the Na,. In the crayfish motor axons, local application of a low concentration of potassium could recover from the propagation failures of axons [63]. The regulatory mechanism of propagation failure by neuronal activity or local environment is an issue to be addressed in future studies.

**Anologue tuning of axonal spike waveforms**

Modulation of action potential duration affect the subsequent transmitter release process at the axon terminals. Although the experimental evidence is limited so far, several studies have demonstrated analogue tuning of axonal spikes at the single axon or single terminal level.

Action potential width at the hippocampal mossy fiber terminals was broadened by the repetitive stimuli at short intervals [19]. It was revealed that this was due to progressive inhibition of voltage-dependent K channels shaping repolarizing phase of the action potentials. Another intriguing example of analogue tuning was demonstrated at the axons of hippocampal CA3 pyramidal cells in culture [22]. Since local application of glutamate caused broadening of axonal action potentials, it will be interesting to test the heterosynaptic interaction from the neighboring axon terminals that release glutamate. It will also be important to study the influence of gliotransmitters released from the surrounding astrocytes.

**Hippocampal mossy fiber as a model of cortical en passant axons**

Hippocampal mossy fiber is an excellent model system for studying physiology of cortical unmyelinated axons. Morphology of the relatively simple structure of en passant axons without extensive branching was described in detail [64, 65]. It was notable that accumulating experimental data of membrane properties and ionic conductance were also available by direct recoding from the axon terminals [5, 19, 20, 31, 39] and from the axonal blebs [24, 29]. Taking advantage of the precise anatomical and physiological data, it is ideal for reconstructing the realistic model of spike propagation along the axons [5, 29, 39].

We recently developed a loose-patch clamp technique from the mossy fiber boutons resolved extracellular unit action potentials of axon, which occur in an all-or-none fashion, with high signal-to-noise ratio. Since extremely large boutons several µm in diameter are readily identified (Fig. 3) under the optics with infrared differential interference contrast (IR-DIC), direct loose-patch clamp recordings from the individual boutons are readily applicable. By quantitative evaluation of the amplitude as well as the time course of “axonal units”, it will be feasible to evaluate the activity-dependent control of the axonal spike, testing on the prevailing notion of digital signaling in the axons. Analogue control of axonal spikes by changes in local microenvironment [23, 63] will be an important issue to be tested.

![Fig. 3 Recording from visualized hippocampal mossy fiber boutons with IR-DIC optics. Low- (left) and high- (right) magnification images of the CA3 region in mouse hippocampal slices. Recording pipette (asterisk) was placed on the large mossy fiber boutons (MFB) located in the stratum lucidum (Luc), inner margin of the stratum pyramidale (Pyr) "](image)
Conclusions

In this review article, we focused on recent progresses in the neurobiological studies of the excitability of axon in the CNS. It is notable that the experimental data on the molecular and cellular mechanisms underlying action potential initiation and propagation in CNS axons are severely limited, although the understanding of excitability tuning of the CNS axons is a fundamental issue to be addressed. Promising approaches are the subcellular patch-clamp recordings from the axons or the axon terminals. Although optical detection of action potentials with new generation of ultrasensitive genetically encoded calcium indicator (e.g., GCaMP6 [67]) or membrane potential indicator (e.g., archaerhodopsin [68]) can detect the spike of single axon terminals in the cultured hippocampal neurons [69], the excellent signal-to-noise ratio as well as ultrafast temporal resolution of the electrophysiological recording is surely needed to study analogue modulation of axonal spikes. Direct electrophysiological recording from the hippocampal mossy fiber boutons will help reveal many important and fundamental issues relating to the local and fine-tuning of the excitability of the axons in the CNS.

Compliance with ethical standards

Conflict of interest The author(s) declare that they have no competing interests.

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