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# ELECTROPHYSIOLOGICAL INVESTIGATION OF THE EFFECT OF GLUTAMATE ON THE PROPAGATION OF ACTION POTENTIALS IN A SINGLE PLANT CELL

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## ABBREVIATIONS

AA – Amino acid

ABA – Abscisic acid

APs – Action potentials

APW – Artificial pond water

ATD – Amino-terminal domain

AtGLRs – Arabidopsis thaliana glutamate-like receptor genes

CAX – Cation/H+ exchangers

CNQX – 6-cyano-7-nitroquinoxaline-2,3-dione

DNQX – 6,7-dinitroquinoxaline-2,3-dione

ER – Endoplasmic reticulum

$E_{th}$ – AP excitation threshold

GABA – Gamma-aminobutyric acid

GHK – Goldman-Hodgkin-Katz equation

Glu – Glutamate

GLR – Glutamate-like receptor channel

GP – Gradual potential

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IP3 – Inositol 1,4,5-trisphosphate

iGluR – Ionotropic glutamate receptor

MAMP – Microbe-Associated Molecular Pattern

MAPKs – Mitogen-activated protein kinases

mGluRs – Metabotropic glutamate receptors

MNQX – 5,7-dinitro-1,4-dihydro-2,3-quinoxalinedione

MP – Membrane potential

MR – Membrane resistance

N-rich – Nitrogen-rich

$[Ca^{2+}]_{cyt}$ – Cytoplasmic calcium concentration

ON – Organic nitrogen

PR – Primary root

P-P – Peak-to-peak time period between action potentials

RD – Repolarization duration

RP – Resting potential

SMC – Soil microbial community

SV – Slow vacuolar channel

TRIS – tris(hydroxymethyl)aminomethane

$V_{prop}$ – AP propagation velocity

# INTRODUCTION

Plants have long been known to respond to their environment by various means, such as changing their growth pattern, producing phytochemicals, altering gene expression. Some of these processes are regulated by the use of electrical signals - action potentials (APs) - and have been observed in both higher plants and algae (Sukhov et al. 2019) (Lunevsky et al. 1983). An AP is a rapid, transient propagating change in the electrical potential along the plasma and vacuole membranes of a cell. APs are initiated in response to a variety of stimuli, including mechanical stress, light and temperature changes, external chemical influences (salinity, heavy metals, amino acids). They can also be artificially induced by applying an electrical stimulus. Once an AP is initiated, it propagates along the length of the cell or the plant tissue. This allows rapid communication enabling systemic responses to stimuli: coordination of growth and defense mechanisms, thus adaptation to changing environmental conditions. Action potential propagation is a fundamental mechanism that enables plants to function and thrive in their surroundings (Sukhov et al. 2019).

The characteristics (amplitude and duration) of APs in plants can be influenced by various chemicals, environmental factors or signaling molecules, such as glutamate (Glu), the most abundant amino acid (AA) found in plants. It plays a crucial role in plant metabolism, in addition, glutamate has been found to act as a signaling molecule in higher plants, comparable to its role in the nervous system of animals (Young and Ajami 2000). The signaling role of glutamate is mediated by glutamate-like receptors (GLRs) permeating $Ca^{2+}$ and other cations into the cell (Davenport 2002). The influx of $Ca^{2+}$ into the cytoplasm initiates activation of specific channels, leading to the modulation of ion fluxes, membrane potential changes, and in some cases generation of APs (Qiu et al. 2020). There is evidence that in higher plants initial stages of electrical signaling are partially mediated by GLRs. This modulation of $Ca^{2+}$ influx into the cytoplasm is important for a variety of plant processes, including growth and response to environmental stresses (Liao et al.2022).

In non-vascular plants such as liverworts, Glu can induce APs with an increased duration and amplitude (Krol et al. 2007). In Characeaen macroalgae - a type of freshwater algae - Glu induces alterations of electrically elicited APs. It hyperpolarizes AP excitation threshold, prolongs AP repolarization and increases AP amplitude (Lapeikaitė 2020). However, it is not known whether the external amino acids alter the propagation properties of APs in plants, also if these alterations of AP parameters upon exposure to AA correspond to or alter the propagation velocity of APs in a single plant cell and in a tissue. In plants Glu signaling provides a framework for investigation of AP

propagation regulation and main electrophysiological factors determining the propagation properties, which directly influence the spatial and temporal characteristics of physiological responses. Characeaen algae - a model system of plant electrophysiology - are well-suited for addressing questions related to AP propagation in a single cell due to their large size (Tabata and Sibaoka 1987).

***The aim of the study:*** to investigate the effect of glutamate on the propagation of APs in a single plant cell.

***The objectives of the study:***

- to determine AP propagation velocity along the intermodal cell of *Nitellopsis obtusa* in opposite directions in standard conditions
- to investigate the effects of exogenous glutamate on the propagation velocity of APs
- to determine whether glutamate induced alterations of AP parameters correspond to the changes of AP propagation velocity

# 1. LITERATURE REVIEW

## 1.1 Electrical signaling in plants

### 1.1.1 General features

Electrical signaling is an important means of communication and response in plants(Fromm and Lautner 2007). Plants generate and propagate electrical signals known as action potentials (APs) in response to external stimuli. In a natural environment, APs are elicited by mechanical irritation,(Kishimoto 1968) (Takao Sibaoka 1991) (Shepherd et al. 2008) cold,(Fromm 1994) (Opritov et al. 2005) (E. Krol et al. 2006) changes in the ionic composition of the medium and as a response to the actions of light or shadow (Trebacz, Dziubinska, and Krol 2006). In laboratory experiments, APs can also be induced by chemical agents (including amino acids), (Felle and Zimmermann 2007) (Elzbieta Krol et al. 2007) (Stolarz, Krol, and Dziubinska 2010) electrical stimulation (Lapeikaitė 2020). Regardless of the origin of the stimulus, the propagation of an AP results in noticeable functional alterations (changes in pH level, chloroplast fluorescence, etc.) ( Bulychev and Komarova 2014)

The observation of APs in Characeaen algae was one of the first instances that led to the field of plant physiology and electrophysiology (Blinks, Harris, and Osterhout 1929) (Beilby 2007). The kinetics of APs in plants depend on the activity of ion channels in the plant cell membranes, a mechanism common to both plants and algae, including Characeae (Okihara et al. 1991). The plasmalemma and tonoplast are membrane systems found in plant cells. The tonoplast is the membrane surrounding the vacuole, which is the largest organelle in plant cells. Both the plasmalemma and tonoplast ion channels contribute to the maintaining of resting membrane potential (MP) and generation of APs (Hilleary et al. n.d.). In plant cells, both the plasmalemma and tonoplast undergo excitation, with tonoplast APs being slower and of smaller amplitude (Kikuyama and Tazawa 1983).

The propagation of APs in plants occurs through conducting bundles of stem beyond the area of its generation. The mechanism of AP transmission from one excitable cell to another is still unknown, but it is suggested that local currents may flow between the activated and adjacent unexcited parts, similar to the conduction of AP along a single excitable cell. The velocity of AP propagation in plants can vary, with differing velocities observed in different species, conditions, directions etc.

### 1.1.2 Membrane potential

The resting MP is the electrical potential difference across the plasma membrane of a cell when it is not excited. It is maintained by the activity of ion channels and transporters in the membrane, which creates an uneven distribution of ions (primarily $K^+$, $Na^+$, and $Cl^-$ in animal cells) across the membrane. The resting MP is negative in animal cells and ranges from -40 to -90 mV, while in plant cells, it can be more negative and ranges from -100 to -180 mV (Hedrich 2012). The membrane resting potential of *Nitellopsis,* a genus of freshwater algae, has been found to be around -180 mV to -200 mV (Lapeikaitė 2020). In plant cells, the resting MP is influenced by the activity of $H^+$-ATPases and $K^+$ channels, which pump $H^+$ ions out of the cell and $K^+$ ions into the cell, respectively. This creates a higher concentration of $K^+$ ions inside (100 mM) the cell and a lower concentration of $H^+$ ions inside the cell. This concentration gradient pulls $K^+$ ions into the cell (Lunevsky et al. 1983).

The resting MP can be calculated using the Goldman-Hodgkin-Katz (GHK) equation. The GHK equation takes into account the permeability of the membrane to each ion and the concentration gradient of each ion.

The GHK equation is used primarily in animal cells, where the resting MP is influenced by the activity of $Na^+$, $K^+$, and $Ca^{2+}$ ion channels. In plant cells, the GHK equation is less applicable, as the resting MP is influenced by the activity of $H^+$-ATPases and $K^+$ channels.

As follows from the equation, the resting MP can be altered by changes in environmental conditions, such as changes ion concentrations, pH, and temperature.

Plant cells can exist in different states of resting plasma membrane, depending on the potassium ion concentration in external media (Hedrich 2012).

1. Under physiological conditions, plant cells can have a plasma membrane potential as negative as 200 mV (when extracellular concentrations of $K^+$ are less than 50 μM). This state is dominated by the P-type proton pump (Beilby and Shepherd 2001).

2. Cells that have MP around -105 mV are considered to be in the steady background cells. They are usually less excitable than “pump state” cells (Beilby and Shepherd 2001).

3. When the $K^+$ concentration increases above 100 μM, the "pump state" is switched into the "$K^+$ state", and the membrane potential follows the Nernst potential of the alkali ion (-91 mV). Opening of $K^+$-channels in the membrane lowers the resistance and provides a current pathway that partially short-circuits the electrogenic pump (Keifer and Lucas 1982).

Intact plasma membrane allows generation and propagation of APs in response to external factors, although various factors can indirectly change the membrane resistance. Those factors include abiotic stresses like drought, salinity and freezing (Beilby and Khazaaly 2017); disruptions in the integrity of the cell wall (Nourbakhsh et al. 2022); changes in the availability of nutrients, such as the concentration of potassium, calcium, and sodium in the soil (Hedrich 2012). Overall, any stress or disturbance that reduces a plant cell's ability to regulate ion flow across its plasma membrane can decrease its membrane resistance. Thus, the membrane resistance of a plant cell is an important factor in its response to environmental stressors and nutrient availability.

Due to constant flow of ions through the plant's plasma membrane the resting potential always fluctuates (Engel et al. 2008). Fluctuations in membrane potential can lead to the generation of action potentials when the threshold voltage is reached.

### 1.1.3 APs in plants

Plants' APs share basics features to those found in animal nervous systems(Garzón and Keijzer 2011). Firstly, they can be induced only by non-damaging stimuli, including electrical currents. Secondly, the generation of APs has an all-or-none character, meaning that they are not induced by sub-threshold stimuli, but rather over-threshold stimuli that produce APs with a constant amplitude (Sukhov, Sukhova, and Vodeneev 2019). Thirdly, APs are self-propagating signals that can propagate through living tissues at a constant rate, usually between 0.1-10.0 cm/s in different plants, but can be up to 10-20 cm/s in plants that exhibit rapid movements (Pyatygin 2006). The propagation of APs does not decrease in velocity or magnitude of amplitude and can only be transmitted down the tissue with low-resistance plasmodesmata connections. Finally, the generation and propagation of APs are limited by absolute and relative refractory periods, with carnivorous plants have a refractory period of seconds, while algae and mosses have refractory periods of a few minutes (Pickard 1973).

The generally accepted model of AP generation involves depolarization of the membrane potential due to calcium influx into the cytosol, followed by $Cl^-$ efflux through $Ca^{2+}$ activated $Cl^-$ channels. Voltage-gated potassium channels are then activated, allowing $K^+$ efflux, which repolarizes the MP. Additionally, ATP-dependent proton efflux also contributes to MP repolarization (Pickard 1973).

The involvement of two membranes, multiple ion transport systems, and the lack of cell specialization account for the variable shape of plants APs compared to animal APs (Beilby and Casanova 2014).

The process of generating APs appears to be similar in both Characeaen algae and higher plants. However, the main contrast between APs in these two plant groups lies in their kinetics and propagation velocity (with Characeaen APs propagating much slower) (Kisnieriene et al. 2022).

#### 1.1.3.1 Mechanism of excitation

The mechanism of excitation is not yet fully understood among plants. While the increase in cytoplasmic calcium concentration ($[Ca^{2+}]_{cyt}$) is known to play a role in AP initiation, the precise origin of $Ca^{2+}$ ions entering the cytosol remains uncertain. Various hypotheses have been proposed, including the entry of $Ca^{2+}$ from the extracellular space or release from intracellular reservoirs. Understanding the mechanisms underlying excitation in plants is a topic of ongoing research and investigation.

*Cytoplasmic* $Ca^{2+}$ *increase*

In plant cells, the increase in $[Ca^{2+}]_{cyt}$ can lead to the initiation of an AP. The origin of $Ca^{2+}$ ions entering the cytosol is presently the subject of ongoing research, and it is uncertain at this time. However, two prominent hypotheses have been proposed to explain this phenomenon: $Ca^{2+}$ ions can enter the cytosol from the extracellular space through the apoplast or from the intracellular reservoirs, such as the endoplasmic reticulum (ER) (Kikuyama, Shimada, and Hiramoto 1993), the vacuoles (Lunevsky et al. 1983), etc (Tazawa and Kikuyama 2003).

For the intracellular origin, $Ca^{2+}$ from vacuoles (Lassalles et al. 1990), membrane vesicles derived from tonoplast (Schumaker et al.1987), microsomes (Canut et al. 1993), mitochondrias (Canut et al. 1993), ER (Guillemette et al. 1988), Golgi membranes and calciosomes (a particular class of light endomembranes in animal systems) (Volpe 1988) is released by the actions of ligand- and voltage-gated channels. This principle has been conserved throughout evolution from bacteria to eukaryotes. The initial ~1000-fold difference between cytoplasmic and non-cytoplasmic $Ca^{2+}$ concentrations enables the generation of calcium signals (Stael et al. 2012).

The central vacuole contains most of the water-soluble $Ca^{2+}$, thus vacuolar $Ca^{2+}$ channels play a critical role in $Ca^{2+}$-mediated signal transduction (Hetherington and Brownlee 2004). Both the $Ca^{2+}$ concentration gradient and the membrane potential drive $Ca^{2+}$ efflux from the vacuole (via $Ca^{2+}$ channels), while $Ca^{2+}$ uptake into the vacuole (mediated by P-type $Ca^{2+}$ pumps (Geisler et al. 2000) and $H^+/Ca^{2+}$ antiporters (Shigaki and Hirschi 2006)) requires energy. Because of the huge cytosol-directed electrochemical gradient for $Ca^{2+}$, the opening of any $Ca^{2+}$-permeable channel will result in $Ca^{2+}$ release from the vacuole (Pottosin and Schönknecht 2007).

To date, at least two ligand-gated $Ca^{2+}$-permeable channels and a voltage-dependent channel have been reported in plant vacuoles. Recently, voltage-dependent channel (so-called slow vacuolar (SV) channel) (Pottosin and Schönknecht 2007) was identified as the product of the TPC1 gene in Arabidopsis and it is by far the best described vacuolar ion channel (Szponarski et al. 2004). Electrophysiological, genetic, and crystallographic studies have revealed that SV/TPC in plant vacuoles is a $Ca^{2+}$- and voltage-dependent, nonselective cation channel permeable to both mono- and divalent cations. In addition to the slow kinetics of its activation (hundreds of ms), it strongly rectifies allowing cation flow mainly from the cytoplasm to the vacuole lumen (Szponarski et al. 2004). Ligand-gated Ca2+ release channels are located mostly in non-vacuolar stores (Muir and Sanders 1997) (Navazario, Mariani, and Sanders 2001) (Martinec et al. 2000). Some calcium imaging experiments have demonstrated that inositol 1,4,5-trisphosphate (IP3) (released from its caged form by photolysis) elicits a transient increase in cytoplasmic calcium by mobilization from internal stores (Gilroy, Read, and Trewavas 1990). A chain of reactions is assumed to occur, involving phosphoinositide-specific phospholipase C (PI-PLC) activation, production of IP3 and IP3-dependent $Ca^{2+}$ release from internal stores (Tazawa and Kikuyama 2003). Second messenger concept could base the all-or-none excitation nature of APs: if the second messenger level remains below a threshold, no $Ca^{2+}$ is mobilized from the stores (vacuole or ER), above the critical value, $IP_X$ causes acute mobilization from the internal stores (Wacke 2003). Keeping in mind the strong evidence that IP3 and ryanodine/cADPR-type receptor $Ca^{2+}$ channels play an important role in $Ca^{2+}$-mediated signal transduction in plant cells, it is surprising that no corresponding genes have yet been identified in plants (Nagata et al. 2004).

For the extracellular origin, a $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$-permeable channels is postulated(White et al. 2002). In addition to depolarization-activated (TPS1) and hyperpolarization-activated $Ca^{2+}$ channels, $Ca^{2+}$-permeable 'voltage- independent' cation (VIC) channels and $Ca^{2+}$-permeable outward rectifying $K^+$ channels have also been observed in the plasma membrane of plant cells (*Arabidopsis*) (White et al. 2002).

The coupling of the two processes may cause complex excitability regulation in Characeaen algae via the so-called calcium-induced calcium release (CICR) - a small portion of $Ca^{2+}$ crossing the plasma membrane from the apoplast would lead to $Ca^{2+}$ release from internal stores (Kisnieriene et al. 2022).

*$Cl^-$ efflux*

The influx of $Ca^{2+}$ then activates $Ca^{2+}$-dependent anion channels in the plasma membrane, leading to an efflux of $Cl^-$ ions from the cytoplasm (Trebacz, Dziubinska, and Krol 2006). This efflux can be mediated by known voltage-dependent R-type anion channel QUAC1 (QUickly activating Anion Channel 1) or S-type $Ca^{2+}$-dependent and voltage-dependent anion channel SLAC1 (Slowly activating Anion Channel 1) (*Arabidopsis*) (Hedrich, Salvador-Recatalà, and Dreyer 2016) (Cuin, Dreyer, and Michard 2018) .

The empirical data indicates that the amplitude of depolarizing $Cl^-$ current is proportional to the square of the cytoplasmic $Ca^{2+}$ concentration in Characeae (Berestovsky and Kataev 2005). Inactivation of Chloride channels (decay of current) was reported to coincide with decreasing calcium(Lunevsky et al. 1983) as it enters the known $Ca^{2+}$ buffering system of Chara (Kikuyama and Tazawa 1983).

*Outward $K^+$ current and activation of H+-ATPase*

The sum of negative feedback on calcium entrance and outward $K^+$ and $H^+$ current is responsible for the repolarization phase of APs (Lapeikaitė 2020).

A negative feedback on calcium entrance in plant cells is explained by the Thiel-Beilby mathematical model for Chara. According to this model, increased calcium concentration in the cytoplasm inactivates calcium channels in the ER, thus imposing negative feedback on calcium entrance (Beilby and Khazaaly 2016).

In high plants, voltage-gated potassium channels GORK (Gated Outwardly-Rectifying K+ Channel) (Cuin, Dreyer, and Michard 2018) (Hedrich, Salvador-Recatalà, and Dreyer 2016) are activated by depolarization of MP (which has been triggered by the efflux of Cl− ions) and are responsible for the efflux of $K^+$ ions from the cytoplasm, resulting in membrane repolarization (Lunevsky et al. 1983). These channels are opened just before the steady-state potential (+95mV)(Wayne and Staves 1991) for $Cl^-$ ions is reached. In Chara, $K^+$-channels were observed to completely inactivate within 20 to 30 s after activation of the $Cl^-$ currents (Lapeikaitė 2020).

The activity of $H^+$-ATPase is re-activated by the decrease in the $Ca^{2+}$ concentration (Sukhov and Vodeneev 2009). Active $Ca^{2+}$ transport from cytosol is mediated by P-type ATPases (Roelfsema and Hedrich 2010), specifically the plasma membrane $Ca^{2+}$-ATPases. These ATPases, belonging to the P-type 2B proteins group, become active at high intracellular $Ca^{2+}$ concentrations, and deactivate at low $Ca^{2+}$ levels (Roelfsema and Hedrich 2010). A second group of $Ca^{2+}$-ATPases, classified as 2A P-type, facilitate $Ca^{2+}$ transport by binding $Ca^{2+}$ ions and undergoing conformational changes upon

ATP hydrolysis. Counter-transport of two $H^+$ ions per cycle influences $Ca^{2+}$ transport. Additionally, high capacity cation/$H^+$ exchangers (CAX) contribute to $Ca^{2+}$ homeostasis by removing excess $Ca^{2+}$ after a $Ca^{2+}$ burst (Roelfsema and Hedrich 2010).

#### 1.1.3.2 Physiological implications of APs

APs in plants are elicited by the variety of inputs, thus their main function is to transmit the information about locally acting stimuli to the distant plant cells (Trebacz, Dziubinska, and Krol 2006).

In high plants, APs in plants facilitate long-distance signaling of information about environmental stimuli such as water availability, light intensity, and herbivore attack (Karban 2008). APs play role in regulation of gene expression, leading to the synthesis of specific proteins that may be involved in stress responses or other physiological processes (Casal and Yanovsky 2004); stomatal regulation, which is important for controlling water loss and gas exchange in plants (Qi et al. 2018); generation of root pressure, which is important for the uptake and transport of water and nutrients in plants (Fromm and Lautner 2007); regulating the growth and directionality of pollen tubes during fertilization (Fromm and Lautner 2007); triggering a wound response in plants, leading to the synthesis of defense compounds and the reinforcement of cell walls in damaged tissues (Fromm and Lautner 2007).

Characeaen algae also generate APs. Physiological implications of APs in Characeaen algae include the regulation of are ion transport and cytoplasmic streaming. During the AP, there is a rapid change in ion concentrations and membrane potential, which leads to the loss of turgor pressure within the cell. This loss of turgor pressure affects various ion transport processes, including the movement of essential nutrients such as nitrate and phosphate (Lunevsky et al. 1983). Cytoplasmic streaming: Characeaen algae have a unique feature called cytoplasmic streaming, in which the cytoplasmic contents flow in a circular motion. APs are involved in regulating the direction and velocity of cytoplasmic streaming. Sudden cessation of protoplasmic streaming (due to the $[Ca^{2+}]_{cyt}$ increment), caused by APs, thought to act as a safety mechanism in the case of cell lesion (Williamson and Ashley 1982).

Many if not all of these physiological processes are fine-tuned by calcium signaling cascades (calcium wave travels together with the AP) (Medvedev 2018). Different environmental/internal stimuli induce cytoplasmic $Ca^{2+}$ oscillations unique in spatiotemporal properties, named calcium signatures, ranging from processes in the immediate vicinity of activated $Ca^{2+}$ channels to large-scale

propagating waves (Medvedev 2018) and the kind of response and its degree depends on the susceptibility of individual cells or tissues to $Ca^{2+}$ fluxes (Trebacz, Dziubinska, and Krol 2006).

#### 1.1.3.3 Alterations of APs in response to environmental stimuli

Typically, APs are uniform signals regardless of the stimuli that generated them (Trebacz, Dziubinska, and Krol 2006). However, in plant cells, APs are more variable than in animal cells. This may be attributed to the excitability of both the plasma membrane and tonoplast in plants (Beilby 2007). The shape of Characeaen APs (defined by temporal characteristics and amplitudes of transient currents and the AP itself) is more variable not only due to this excitability, but also due to the changes in physical conditions and environmental factors (Lapeikaitė 2020). Those factors include:

– "Shelf life" affects the characteristics of *Nitellopsis* cell membranes (Lunevsky et al. 1983).

– Temperature affects AP parameters, including duration, amplitude, and refractory period as well as Cl- current transients. For instance, decreased temperature (from 40°C to 3.5°C) causes increased duration of both membrane and tonoplast AP (Beilby and Coster 1976a) as well as decreased amplitude and refractory period in Chara (Beilby and Coster 1976b).

– Salinity alters one of the most variable parameters of AP in Characeae (Kisnieriene et al. 2012) - repolarization rate. Mild salinity (50 mM NaCl) effects AP shape and extended the AP duration (from 2 to up to 60 s) (Kisnieriene et al. 2017). High salinity (100 mM NaCl) alters AP threshold and doubles durations of AP (Beilby and Khazaaly 2017).

– Exposure to various substances has a profound effect on AP parameters in Characeae. For instance, heavy metals increase the AP peak value, prolong the slow phase of repolarization and decrease $Cl^-$ and $Ca^{2+}$ effluxes during excitation (Kisnieriene and Sakalauskas 2005) (Sevriukova et al. 2014).

– Light, heat, and cold can also induce APs of longer durations in comparison to electrically induced APs (Favre et al. 1999).

– Signaling molecules: acetylcholine and Glu increase the duration of AP repolarization in cells in a $K^+$ state (probably due to increase $Cl^-$ channel open probability in the tonoplast of Chara (Gong and Bisson 2002)), depolarizes MP and leads to spontaneous generation of APs (Kisnieriene et al. 2012).

– AAs induce APs in in plant cells (Felle and Zimmermann 2007) (Krol et al. 2007) and significantly increase the half-time duration of triggered APs (up to 3-fold) and heightens its amplitude (Krol et al. 2007).

– Asparagine as well as glutamate excites plant cells and causes the modulation of AP shape (Lapeikaitė 2020).

## 1.2 AP propagation

### 1.2.1 AP propagation in higher plants

AP propagation in plants is a process by which an excitation travels across the length of a plant cell (Pickard 1973). Effect of propagation is created when local depolarization reaches the threshold level and APs are generated in the region next to the previously excited one (Pyatygin 2006). On the other hand, AP transmission in plant cells refers to the transfer of electrical signals between cells. It is a complex process that involves the movement of ions across the plasma membrane and through plasmodesmata, and plays a critical role in plant signaling and communication (Pickard 1973).

AP generation in higher plant cells occurs due to the passive flux of ions through channels and a transitional change of the electrogenic activity of an ATP-dependent $H^+$-pump ($H^+$-ATPase) of the plasma membrane following an "inactivation-activation" pattern. The operational characteristics of ion channels in plants are likely slower than those in animals, affecting the speed of AP generation. However, rapid AP propagation in these plants is supported by a high coefficient of electric coupling between pathway cells, which can reach 0.8 (high degree of electric coupling, signifying efficient electrical communication and transmission of signals within the plant's vascular system) in some species of vascular plants (Sukhov and Vodeneev 2009).

In higher plants, AP propagates along the conducting bundles of stem beyond the area of its generation. Since the phloem sieve-tube system consists of a continuous plasma membrane, it could potentially serve as a pathway for transmitting the electrical signal. For example, excitable cells which generate an AP and serve as the path of transmission in the petiole of *Mimosa* are found in the vascular bundles; the elongated parenchyma cells in protoxylem and phloem are excitable, and they form a number of cell rows along the longitudinal axis (Sibaoka 2003). The phloem is capable of conducting bioelectrochemical impulses over significant distances, making it an effective form of long distance communication between plant tissues and organs (Volkov 2000). Based on the size of excitable cells and transmission velocity, it is estimated that each excitable cell in a particular cell row in the *Mimosa*

petiole generates an AP successively with an interval of 4 to 6 ms (Sibaoka 2003). There are differing views on how APs propagate over the root and leaf: directly or as a transduced signal or whether they act via a series of mediators. There is good evidence to believe that the AP transmitted along the stem of higher plants is a nonspecific bioelectric signal independent of the stimulus origin; consequently, it does not carry any stimulus-specific information (Pyatygin, Opritov, and Vodeneev 2008). The mechanism of the transmission of the AP from an excitable cell to another remains unestablished. Studies show that the transmission from cell to cell may be mediated by local current which flows between the just activated part and the adjacent yet unexcited part (similar mechanism to the conduction of the AP along a single excitable cell) (Sibaoka 2003).

Being a nonspecific bioelectric signal, the transmitted AP induces transient nonspecific functional disturbances in tissues and organs (leaves, roots, ovary, etc.,) on the way of its transmission. Once AP is initiated in higher plants, it spreads across the stimulated region and can travel in multiple directions. Interestingly, the velocity differs between the first and subsequent APs in *Mimosa*, with the second action potential exhibiting a faster transmission. Notably, in carnivorous plants, the velocity of action potential transmission is faster parallel to the vascular bundles compared to the perpendicular direction. Higher number of excitable cells in the tissue also positively correlates with the action potential transmission velocity, because it provides more pathways for the transmission of electrical signals. When there are more excitable cells available, the action potential can be transmitted through multiple parallel pathways, allowing for faster and more efficient signal transmission (Krol et al. 2006).

In conclusion, the transmission velocity depends on the coefficient of electric coupling between pathway cells (the phloem sieve-tube system serving as a pathway for transmitting the electrical signal), direction and the number of excitable cells.

### 1.2.2 AP propagation velocity

Attempts to calculate AP propagation velocities using a detailed mathematical model of plant electrical signal transmission have been made for a single plant cell (Pyatygin 2006) and for plant tissue (Sukhov et al. 2011).

Theoretical calculations of AP propagation velocities in plant cell have been successfully modeled using cable theory. Cable theory provides a framework for understanding the mechanism of AP propagation in the cytoplasm of plant pathways and draws parallels to the propagation in nonmyelinated nerve fibers of animals. This similarity is attributed to the shared cable properties of

excitable pathways, including excitation threshold, membrane capacity, and external and internal resistances. By applying cable theory, propagation velocities of APs in higher plants were mathematically predict, aligning well with experimental findings (Pyatygin 2006).

$$V_{prop} = \lambda \sqrt{\frac{dU/dt}{\Delta U_{th} \cdot R_m \cdot C_m}}$$

Where:
λ - cable constant (a parameter that describes the spatial decay of the electrical signal along the plant cell);
dU/dt - the steepness of the pulse rise-up portion at the moment when the excitation threshold is reached;
$\Delta U_{th}$ - the difference between membrane resting potential and action potential $E_{th}$;
$R_m$ - membrane resistance;
$C_m$ - membrane capacitance;
$R_m$ = the membrane resistance;
$C_m$ = the membrane capacity;

Another mathematical model of action potential transmission in plants is a two-dimensional system of excitable cells; each of them is electrically coupled with four neighboring ones (ion diffusion between excitable cell apoplast areas is taken into account). The model shows that the cell to cell electrical conductivity and the plasmalemma $H^+$-ATPase activity influence AP generation and transmission. In particular, it suggests that sieve elements possessing the high electrical conductivity between cells and the low plasma membrane $H^+$-ATPase content can be a good system of the AP transmission, but not optimal for the AP generation. A rise in electrical conductivity between cells leads to an augmentation in the length constant (λ), action potential transmission velocity, and stimulus threshold. On the other hand, an increase in $H^+$-ATPase activity results in an elevation of the stimulus threshold, reduced length constant and slowed transmission (Sukhov et al. 2011).

The spread in reported AP transmission velocity values in the literature can reach several orders of magnitude, suggesting that higher plants may possess APs of different velocity types, ranging from relatively slow to paradoxically fast ones. Attempts have been made to classify APs into three types based on their duration and propagation velocity (Pyatygin 2006).

The first type is "slow" APs, which are registered in the stem and some other organs of common plants that lack specialized locomotor functions. The velocity of slow APs ranges from 0.1 to 1 cm/s, with a lower limit of tenths of a millimeter per second, and their duration is relatively long, several tens of seconds (Pyatygin 2006).

The second type comprises "fast" APs with transmission velocities ranging from tens to hundreds of millimeters per second. They are typical primarily of a limited group of higher plants with specialized locomotor functions, such as *Mimosa* and *Neptunia*, but are also found in some "common" higher plants. The duration of fast APs ranges from fractions of a second to tens of seconds (Pyatygin 2006).

The third group comprises "ultrafast" APs with extremely high transmission velocities, ranging from 500 mm/s to many meters per second, which is more typical of APs in animal nerves. The duration of ultrafast APs in plants is short, ranging from tenths of a millisecond to a few tens of milliseconds, making them close to a nerve pulse. They are the most short-lived APs in higher plants and have been found only in a limited number of "common" higher plants (Pyatygin 2006).

Despite a wide range of reported values, it was acknowledged that the different velocity types of APs might be due to the methodological issues. The knowledge in this area remains scarce and inconsistent. The factors that could potentially contribute to the AP propagation and transmission have to be studied.

In summary, the propagation velocities of APs in higher plants demonstrate a definite dependence on species and plant's individual peculiarities. At the same time, the propagation velocities of APs in a single plant can vary depending on the nature of the stimulus and the location of propagation. The duration of an AP in plants is primarily determined by the efficiency of ion-transporting mechanisms, while the propagation velocity is influenced by the pathway cable properties.

### 1.2.3 AP propagation in Characeaen algae

APs are generated in Characeae in response to changes in temperature, light intensity, and salinity (Kisnieriene et al. 2022). It has been established that the APs peak values recorded in *N. obtusa* cells are typically positive, and the reversal potential of the anion efflux is around 100 mV(Kisnieriene et al. 2017) (Lapeikaitė 2020). Propagation of APs with similar amplitude and temporal parameters in Characeaen algae happens independently of the stimulation strength. The AP is conducted in both directions away from the site at which the plasma membrane is depolarized over the threshold. In *Chara braunii* mean propagation velocity of 1–2 cm/s has been established, and unidirectionality conditioned by channel inactivation has been confirmed (Tabata and Sibaoka 1987). In that study, the conduction velocity of the action potential was measured in two different environments: where part of the cell was exposed to moist air while the other part remained in artificial pond water (APW). In

moist air, the conduction velocity was found to be 0.21 cm/s ± 0.05 cm/s, while in APW, it increased to 1.5 cm/s ± 0.9 cm/s. Interestingly, the action potential propagated at an almost constant velocity along the cell in moist air, except within 0.3 cm from an end of the cell. However, in APW, the velocity showed a significant increase, reaching 5.7 cm/s ± 2.3 cm/s within 1.8 cm from the end of the cell. Conduction velocity was calculated by measuring the time lag between the peaks of the conducted action potential and the distance between the corresponding recording electrodes. Overall, it can be concluded that in *Chara braunii* the conduction rate is dependent on the conductivity of the medium (Tabata and Sibaoka 1987). The lower ability of the cell to conduct corresponds with a slight depolarization of the threshold and an increase of membrane resistance (Tabata and Sibaoka 1987).

It has been shown that Characeaen cells exposed to illumination arrange plasma-membrane $H^+$ fluxes in coordinated spatial patterns thus generating alkaline versus acidic zones just outside of the cell in the surrounding solution. AP propagation together with circulation of electric currents between functionally distinct cell domains and continuous cyclical cytoplasmic streaming effects the photosynthetic activity in Characeaen algae. A single AP enhances the banding patterns in the chloroplast layer of cells while suppressing the banding pattern of extracellular pH. AP effects on the plasma membrane $H^+$ transport and on chloroplast functional activity are mediated by different pathways having in common only the initial step, i.e., a 10- to 100-fold increase in cytosolic $Ca^{2+}$ concentration during AP. After the generation of the first AP membrane conductance decreases in the alkaline cell regions where it is particularly high, thus the propagation velocity of the following AP is also decreased. This decrease in membrane conductance is attributed to the suppression of passive $H^+$ flows (Krupenina et al. 2008). These changes in membrane conductance, which are unique to alkaline zones, were solely detected when cells were continuously exposed to light after a rest period of at least 15 minutes from the previous stimulation. No changes in conductance were observed in the post-excitation period when cells were adapted to darkness (Bulychev and Krupenina 2009).

The results of the studies investigating the effects of pharmaceutical calcium channel blockers (Verapamil, Tetrandrine, NED-19) on the APs generation and propagation provide insights into the molecular basis of APs (Koselski et al. 2021), supported by recent decoding of *Chara braunii* (Nishiyama et al. 2018) and *Marchantia polymorpha* (Bowman et al. 2017) genomes (in 2018 and 2017 respectively). Verapamil and Tetrandrine induced the generation of spontaneous APs in *N. obtusa* that conduct at a slower pace, but at a higher frequencies compared to the electrically induced APs. This suggests that the pharmaceuticals alter the normal electrical activity of the cells, leading to the generation of APs that occur more frequently but propagate at a slower rate (Koselski et al. 2021).

Some experiments also show that when the cells are subjected to repeated excitation at a higher frequency, the AP propagation velocity may decrease. For instance, electrical stimulation at a higher frequency decreases the AP propagation velocity in *N. obtusa*, suggesting that the cell did not reach its steady pre-excitation state (to a condition where the cell has achieved a stable and balanced physiological state before being stimulated). This means that the cytoplasmic $Ca^{2+}$ concentration was not reduced to steady state levels (Koselski et al. 2021).

Inhibition of $Ca^{2+}$ fluxes (caused by Verapamil and Tetrandrine or repeated excitation) which are responsible for the depolarization phase of APs in plants may be the reason of slower AP propagation velocity. Failure to reduce the cytoplasmic $Ca^{2+}$ concentration to steady state levels disrupts calcium dynamics, interfering with the balance of ion currents involved in AP propagation and influencing the opening and closing of ion channels, thereby affecting the electrical excitability of the cell. This can lead to alterations in the timing and magnitude of membrane depolarization and repolarization, subsequently affecting the velocity at which the AP travels along the cell.

In conclusion, APs in Characeaen algae have similar mechanisms of generation to higher plants, but varying kinetics and lower propagation velocities (in general). The conduction rate is dependent on the conductivity of the medium, channel activity (particularly $Ca^{2+}$ fluxes), passive $H^+$ flows (resulting in decreased membrane conductance). The Characeaen algae exhibit a mean propagation velocity of APs, which is estimated to be between 1 to 2 cm/s, and it is conducted in both directions away from the point at which the plasma membrane undergoes depolarization beyond the threshold. The velocity of AP propagation in plants can be influenced by various compounds that affect the dynamics of calcium ions and disrupt ion currents involved in membrane depolarization and repolarization.

## 1.3 Glutamate and its signaling role in plants

### 1.3.1 Glutamate: its structure, homeostasis and role in plant metabolism

Glutamate (Glu) is the most ubiquitous amino acid in nature and has diverse biological roles as a metabolite, energy substrate, nutrient, structural determinant in proteins, and as a signaling molecule. Glutamate has a number of chemical properties that make it particularly well suited to its multiplicity of functions (Young and

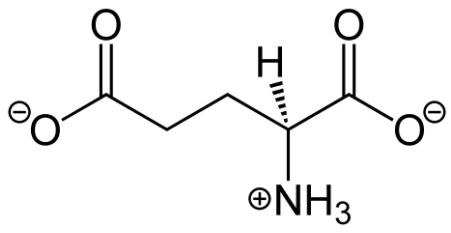


**Fig. 1.1** L-Glutamate structural formula

Ajami 2000). Glutamate is an alpha-amino acid that has a carboxyl group (-COOH) at one end and an amino group (-NH2) at the other end. In prokaryotic cells glutamate exist only in its L-form (**Fig. 1.1**). The amino group of L-glutamate is attached to an alpha carbon atom, which is also bonded to a hydrogen atom and a side chain. The side chain of glutamate is a carboxylic acid group (-CH2-COOH) that is negatively charged at physiological pH. This unique structure gives glutamate its ability to act as a neurotransmitter in animals and a signaling molecule in plants (Young and Ajami 2000). The carboxylic acid group can be easily ionized at physiological pH, resulting in a negatively charged form. This charge allows glutamate to interact with and activate specific receptors, such as the ionotropic glutamate receptors. The amino group contains a positively charged nitrogen atom, which contributes to the overall polarity of the molecule. This polarity enables glutamate to participate in electrochemical signaling by promoting the influx of cations.

Glutamate is a central molecule in plant metabolism and is involved in numerous biochemical pathways. One of its key roles is as a precursor for the biosynthesis of other amino acids. The a-amino group of glutamate participates both in the assimilation and dissimilation of ammonium ($NH4^+$). It can be transferred to other amino acids by various amino transferases. In addition to its role in amino acid biosynthesis, glutamate is also involved in the biosynthesis of proteins and nucleotides (**Fig. 1.2**). Glutamate is also a precursor for the biosynthesis of the neurotransmitter gamma-amino butyric acid (GABA), which plays a crucial role in plant stress responses. Furthermore, glutamate is involved in the regulation of plant growth and development. It has also been implicated in the regulation of seed germination and the response to abiotic stress, such as drought and salinity. Finally, glutamate is a signaling molecule in plants and can induce APs in plant cells (some of the high plants (Forde and Lea 2007) and Characeae (Lapeikaitė 2020)).

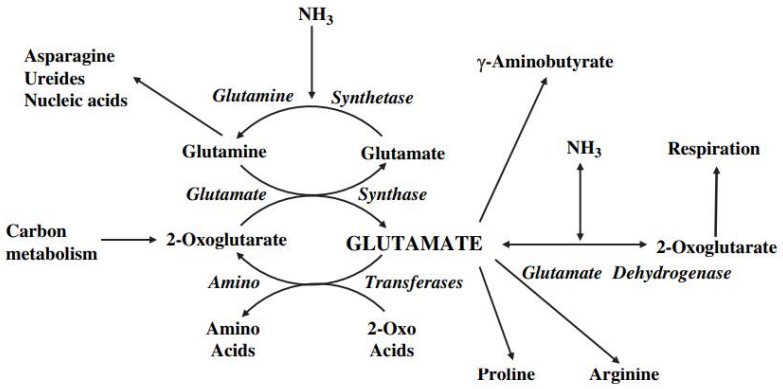


**Fig. 1.2.** Pathways of glutamate synthesis and metabolism in plants; (*credits Brian G. Forde , Peter J. Lea, Glutamate in plants: metabolism, regulation, and signaling, Journal of Experimental Botany, Volume 58, Issue 9, July 2007, Pages 2339–2358, https://doi.org/10.1093/jxb/erm121)*

In plant tissues glutamate concentration is maintained relatively constant during a diurnal cycle, whilst concurrently other amino acids can change dramatically. The reported concentration of Glu in

the cytoplasm of tobacco leaves is 3 – 4 μmol $g^{-1}$ FW; of potato leaves is 1.0–1.3 μmol $g^{-1}$ FW; of Arabidopsis leaves 2 nmol $mg^{-1}$ FW (Forde and Lea 2007). The synthesis of Glu in plants from ammonia occurs primarily in the chloroplasts of photosynthetic tissue and plastids in non-photosynthetic tissue via the glutamine synthetase cycle. Glu is catabolized in mitochondria or cytoplasm via Glu dehydrogenase. Additionally, plants can produce Glu through alternative pathways, such as the Pro/pyrroline 5-carboxylate cycle and transamination (Seifi 2013). Besides, Glu can be taken up directly by the roots, and then transported among tissues or organs through xylem and phloem. In general, the redistribution of Glu can be achieved by transporters locating in the plasma membrane (Qiu et al. 2020). The combination of these processes accounts for maintaining of the glutamate homeostasis.

### 1.3.2 Glutamate in the environment

Plants, animals, and soil microbial community (SMC) contribute the majority of organic nitrogen (ON) to soils in the form of AAs (including Glu), peptides, and proteins. Recent studies suggest that plants can take up AAs and other low molecular weight ON compounds and compete with the soil microbial community for such resources (Knowles et al. 2010). Soil biota hydrolyze proteins to release AAs and low molecular weight peptides that can cross cellular membranes (Knowles et al. 2010).

Amino acids, including Glu, have potential value as a direct source of nitrogen and as a source of information about the location of an organic Nitrogen-rich (N-rich) patch of soil. Significant concentrations of glutamate are likely to be found in N-rich layers of soil, particularly in habitats where mineralization is slow due to low temperatures (Forde and Walch-Liu 2009). However, the chemical complexity of the soil makes it difficult to distinguish which external signals have stimulated root proliferation. The diffusion coefficient of amino acids in soil is estimated to be about 1 mm per day, which is much slower than diffusion coefficient of nitrate (10 mm/day). When plants encounter a localized source of Glu, there is a focused increase in root branching, which is interpreted as a more appropriate response to an organic N-rich source than the expansive response elicited by nitrate. The inhibition of primary root (PR) growth by Glu, combined with nitrate's ability to antagonize this response, enables a plant to modify its root architecture according to the relative abundance of inorganic and organic N in the soil. In low-fertility soils where significant amino acid concentrations may occur in surface layers of organic matter, the relative abundance of Glu and nitrate could be such

that PR growth is inhibited, leading to a shallower, more branched root system that can efficiently exploit the available nitrogen.

In aqueous systems, glutamate is responsible for the uptake of Cu(II), which is essential for many enzyme functions in plants (Fitts et al. 1999).

The studies measuring the uptake of AA in by microorganisms in freshwater lakes in North America found that the uptake velocity varied seasonally and was proportional to temperature (Burnison and Morita 1974). Glutamate, asparagine, aspartate, and serine were the most actively respired amino acids (up to 63% of the amino acids were took up). Glutamate uptake (ranged from 0.44 to 2.32 mumol of C/liter per day) varied seasonally. In addition, polluted sites had higher levels of glucose and glutamate incorporation and higher maximum uptake velocity (Vmax) values (Carney and Colwell 1976).

Glutamate is a naturally occurring compound found in many living organisms, and it can be released into the environment through various processes, such as the decay of plant and animal matter or the discharge of wastewater. Whether or not there is free glutamate present in a particular lake would depend on many factors, including the surrounding ecosystem, nearby human activity, and the specific sampling location within the lake. To determine if there is free glutamate in a specific lake, it would be necessary to collect and analyze water samples from that location. For instance, concentration of glutamate in subalpine Castle Lake, California was reported to range from 0.72 μM to 9.59 μM in different days (Zehr, Axler, and Goldman 1985).

### 1.3.3 Glutamate as a signal molecule

L-Glutamate is now well established as an important signaling molecule in the mammalian central nervous system and glutamate signaling may also occur in higher plants(Forde and Lea 2007). There are evidence, suggesting that both autocrine/paracrine, endocrine and exocrine signaling can take place simultaneously (Qiu et al. 2020). For the exocrine signaling, the reported range of concentrations for apoplastic glutamate in various tissues and species is 0.3-1.3 mM. In studies, conducted on cucumber and Arabidopsis hypocotyls (Zimmermann et al. 1999), was found that glutamate added exogenously to plant cells elicits rapid membrane depolarization and a transient increase in $[Ca^{2+}]_{cyt}$, which effects root architecture, seed germination, pollen tube growth (Liao, Chung, and Hsieh 2022) and adaptation to salt, cold, heat, drought, pathogen, and wound stress (Qiu et al. 2020).

The root system of a plant accounts for 80% of its entire mass and serves as an anchor and nutrient uptake and transport system. It is well described that externally applied Glu elicits specific changes in root system architecture. Exogenously applied Glu recovered root growth that has been impaired by previous application of ionotropic glutamate receptor (iGluR) competitive antagonists; recovered root elongation (inhibited by $Ca^{2+}$ chelator) and lateral root density (inhibited by polar auxin transport inhibitor). There are strong evidence of a synergistic effect of $Ca^{2+}$, auxin, and Glu signaling in root architecture (Qiu et al. 2020).

$Ca^{2+}$ is an essential signaling molecule in seed germination and enters the cytosol through $Ca^{2+}$ channels. Increased cytosolic $Ca^{2+}$ concentration promotes seed germination by counteracting the inhibitory effect of phytohormone abscisic acid (ABA). Application of L-Glu alleviates salt-induced inhibition of seed germination by mediating ethylene production (Cheng et al. 2018)

For pollen germination and tube growth increase in cytosolic free calcium concentration is a fundamental signaling event. Inhibitors of iGluR have an inhibitory effect on $Ca^{2+}$ signaling and pollen tube growth, whilst exogenously applied glutamate has an opposite effect(Qiu et al. 2020). Application of AA enhanced the $[Ca^{2+}]_{cyt}$ gradient toward subapical zone in pollen tubes, leading to an inhibition of pollen tube growth (Michard 2011).

Glu is proposed to act as damage-associated molecular pattern (DAMP) – tissue wounding could be indicated by Glu passively leaking or being actively released. Increased apoplastic Glu elicits propagating systemic defense signal through altered $[Ca^{2+}]_{cyt}$ (Lapeikaitė 2020).

Plant immunity is achieved by the recognition of pathogens through the binding of distinct molecules from the pathogen itself or concomitant substances to the receptors (located in the plasma membrane or within the cytosol). Glutamate plays an important role in the immune response by desensitizing ion channels that are associated with Microbe-Associated Molecular Pattern (MAMP)-induced responses, and by activating several mitogen-activated protein kinases (MAPKs) swiftly. Furthermore, glutamate could function as a second messenger for MAMP-induced downstream events (Seifi 2013).

Summarizing, glutamate has diverse biological roles due to its chemical properties. It is involved in various metabolic pathways in plants, serving as a precursor for biosynthesis and acting as a signal molecule. It can elicit membrane depolarization and calcium signaling in plant cells. Glutamate concentration is relatively constant in plant tissues, and its synthesis occurs primarily in chloroplasts via the glutamine synthetase cycle, but it can also be taken up directly by roots and transported among

tissues through xylem and phloem. Glutamate signaling affect root architecture, seed germination, pollen tube growth, and adaptation to various stresses.

## 1.4 Glutamate receptors

### 1.4.1 Abundance of glutamate receptors

Across different kingdoms of life, glutamate receptors are widely present and serve important functions in cellular communication and signaling.

In mammals, iGluRs are non-selective cation channels that operate primarily as glutamate-gated $Na^+$ and $Ca^{2+}$ influx pathways at neuronal (vertebrate) or neuromuscular (invertebrate) junctions. They are classified into four groups based on their sensitivity to agonists: AMPA, NMDA, kainate, and delta receptors. AMPA and kainate receptors exhibit low $Ca^{2+}$ permeability, whereas NMDA receptors are $Ca^{2+}$-permeable and have a slower desensitization rate.

In invertebrates, homologues of mammalian iGluR subtypes are present. Fish, birds, and amphibians express low molecular weight kainate-binding proteins (KBPs), which are similar in sequence to iGluRs and bind glutamate with high affinity, but do not operate as ion channels due to the lack the long N-terminus. In bacteria (*cyanobacterium Synechocystis*), one glutamate receptor (GluR0) has been discovered. GluR0 is $K^+$-selective and is activated by glutamate and glutamine (Davenport 2002).

Plants have a large family of genes encoding GLR. *Arabidopsis thaliana* genome had been fully sequenced, revealing that the plant has 20 GLR genes (*Arabidopsis thaliana* glutamate-like receptor [AtGLRs]) that can be grouped into three clades: GLR1, GLR2 and GLR3 with some authors suggesting a fourth subfamily (GLR4) (Chen et al. 2016). It was hypothesized that all the GLRs have their origins in the GLR0 plant subfamily, which is closely related to the GLR3 subfamily (Chen et al. 2016). The AtGLRs family appears to have developed by multiple local gene duplication events, as indicated by the high frequency of occurrence of adjacent pairs and triplets of very similar GLR genes. It is possible that some of these genes have no function. There is at least one GLR pseudogene in the *Arabidopsis* genome (Davenport 2002). The GLR genes in plants are believed to encode ion channels that have a close structural and sequence similarity to iGluRs found in animals. Based on a phylogenetic analysis of iGluRs from metazoans, plants, and bacteria, it was found that plant GLRs are not more closely related to metazoan iGluRs than they are to bacterial iGluRs. This suggests that

the divergence of plant GLRs from other eukaryotic and bacterial GLRs may have occurred as early as the last universal common ancestor(Price, Jelesko, and Okumoto 2012).

### 1.4.2 Structure of iGluR and GLR

Plant GLRs consist of the same basic structures as animal iGluRs: the channel-forming domain, the ligand-binding domain and an additional modulatory region (**Fig. 1.3**) (Davenport 2002). The mammalian iGluRs are thought to function as heteromers. The channel-forming domain in iGluR consists of three complete trans-membrane domains (M1, M3, M4) that form tetramers or possibly pentamers, with each subunit containing a partial trans-membrane domain (M2) that forms a hydrophobic pore-loop structure, lining the pore region that confers ion selectivity on the channel ($Na^+$, $K^+$, and $Ca^{2+}$) (Davenport 2002).

Two lobes (GlnH1 and GlnH2 or S1 and S2), separated by the first two transmembrane domains and pore region, are forming a ligand-binding domain that regulates channel gating through agonist-induced conformational changes. Additional amino-terminal modulatory domain (ATD) can regulate channel activities by binding a range of ions and molecules. A long N-terminal domain has an unknown function with homology to the ligand-binding domain of metabotropic glutamate receptors (mGluRs), $GABA_B$ receptors, and extracellular $Ca^{2+}$ sensors.

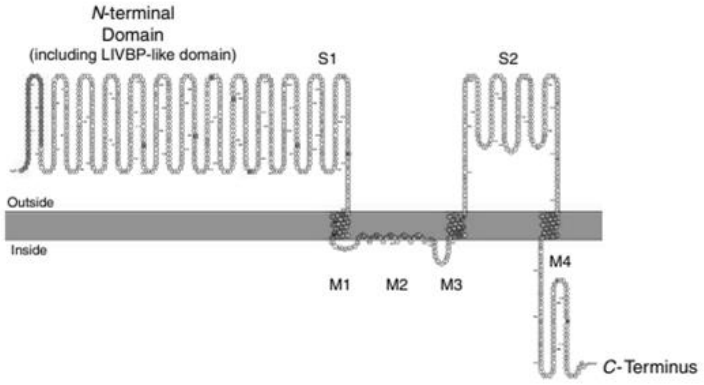


**Fig. 1.3** Structure of GLR, *(credits Wieland et all, 2015)*

Recent genetic evidence suggests that plant GLRs may function as non-selective cation channels, but some subunits or subunit combinations may show sensitivity to ligands with stimulated GLRs undergoing endocytosis and/or degradation (Dietrich et al. 2010). Plant GLRs retain the expected channel structure of animal iGluRs and have significant similarity in their ligand binding domains and transmembrane domains M1 and M2 with some exceptions (absence of P, K/R residues at the beginning of M2). A notable difference between plant GLRs and animal iGluRs is in the putative pore region, where plant GLRs have cationic residues in the 'GYGD' region, which is predicted to interact with the permeant cation and affect selectivity. This feature suggests either a novel selectivity

or a novel mechanism of selectivity for plant GLRs. Similar to iGluR, plant GLRs are anticipated to possess an extended N-terminal region whose function remains unidentified. This region is believed to share similarities with the ligand-binding domain of mGluRs, $GABA_B$ receptors, and extracellular $Ca^{2+}$ sensors (Davenport 2002). GLRs act as ion channels and conduct many cations into the cell in the presence of extracellular amino acids, such as $Ca^{2+}$. The ligand-binding specificity in animal iGluRs is usually determined by the interaction between its subunits. In plants, on the other hand, some GLR subunits could interact with multiple other GLRs, and unlike iGluRs, the sequence similarity between subunits did not necessarily correlate with the likelihood of interaction(Price, Kong, and Okumoto 2013).

AtGLRs are localized in various membranes, including the plasma membrane, chloroplasts, and mitochondria. They play a role in intracellular signaling and can release calcium from internal stores. Fluorescent tagging confirms their presence in the plasma membrane, plastid membranes, and stromules of Arabidopsis (Weiland et al. 2015).

### 1.4.3 GLR mechanism of activation and physiological implications

*Mechanism of activation*

GLR receptors are activated by ligand binding to two ligand-binding domains on the external site of the membranes. The attachment of the ligand molecule leads to conformational changes that cause a closing of the ligand-binding sites, evoking alterations near the receptor pore region and allowing a passage/blockade of ions (Weiland et al. 2015). Receptors can exist in three states:, active state being responsive to ligand binding, inactive state or desensitized state where the ligand is still bound but no other actions can take place (Weiland et al. 2015). Proper protein targeting, including phosphorylation and interaction with 14-3-3 proteins, is crucial for receptor recycling or degradation (Weiland et al. 2015).

Plant GLRs are capable of binding different ligands depending on the composition of the four subunit-comprising receptor complex. With 20 GLRs in *A. thaliana* and each subunit capable of binding two particular ligands, the number of possibilities for variant receptor complex compositions is enormous and helps explain their involvement in many physiological aspects. However, classical iGluR agonists like NMDA, AMPA, or kainate do not have any effect on GLRs in Arabidopsis and are completely absent in plants. This is probably because these agents are not amino acids and do not exhibit an amino acid-related structure (Weiland et al. 2015). The true agonists of GLRs are still not fully understood. Glutamate is often seen as the main agonist due to structural similarity to iGluRs,

despite apparent differences in the ligand-binding sites between these two receptor types. Different GLRs are targeted or activated by distinct amino acids, and competitive antagonism of amino acids against others seems to exist in GLRs (Weiland et al. 2015).

*GLR physiological implications*

Certain AtGLR receptors are involved in regulating C and N metabolism and act as glutamate-gated $Ca^{2+}$ channels. Preliminary evidence from pharmacological approaches using agonists and antagonists of mammalian iGlu receptors, such as 6,7-dinitroquinoxaline-2,3-dione (DNQX), 5,7-dinitro-1,4-dihydro-2,3-quinoxalinedione (MNQX), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and $La^{3+}$, also suggest the involvement of GLRs in $Ca^{2+}$ responses. Some of the confirmed physiological roles related to the $Ca^{2+}$ signaling of GLRs include: AA-regulated intracellular signaling and metabolism (Price, Jelesko, and Okumoto 2012), initiation of lateral root primordial and adjusting of proper root branching (Qiu et al. 2020), plant's defense response, particularly in response to pest and pathogen attacks (Forde and Roberts 2014), defense response to mechanical wounding (Forde and Roberts 2014), mediating of developmental processes (reproduction, senescence) (Weiland et al. 2015), mediating photo- and gravitropism (Weiland et al. 2015), plant stress signaling response in the form of altered gene expression of several GLRs (Weiland et al. 2015), cold stress response (Zheng et al. 2018), sensing toxic environments (Weiland et al. 2015), mediating abscisic acid signaling (Weiland et al. 2015).

Despite their diverse physiological implications, plants GLRs seem to be involved only in the initial steps of $Ca^{2+}$ signaling by mediating the first transient $Ca^{2+}$ influxes. Ligand-evoked currents show that the application of an agonist like L-glutamate does not cause a simple depolarization of the corresponding membrane due to an efflux/influx of a single sort of ions. Instead, the progression of a glutamate-induced $Ca^{2+}$ influx features some distinct stages. At the beginning of a GLR agonist treatment, a strong and rapid $Ca^{2+}$ influx occurs, which leads to increases of $[Ca^{2+}]_{cyt}$. The actual plasma membrane depolarization seems to be a dose- and threshold-dependent event. GLRs are likely to become desensitized upon sequential ligand applications, and a second agonist treatment results in a reduced depolarization amplitude (Weiland et al. 2015). The presence of glutamate alters the initial stages of APs, but their specific role in the propagation velocity of APs is not well-established. Membrane depolarization and subsequent ion influx during action potential generation can impact the propagation velocity of the AP along the plant cell.

## 2. MATERIALS AND METHODS

### 2.1 Object

Internodal cells of mature freshwater macroalgae *Nitellopsis obtusa* (Characeae), commonly known as stonewort, were used as a model system (**Fig. 2.1**). The morphology and physiology of this specie offer a robust experimental system to explore plant electrophysiology using intracellular microelectrode techniques(Kisnieriene et al. 2022).

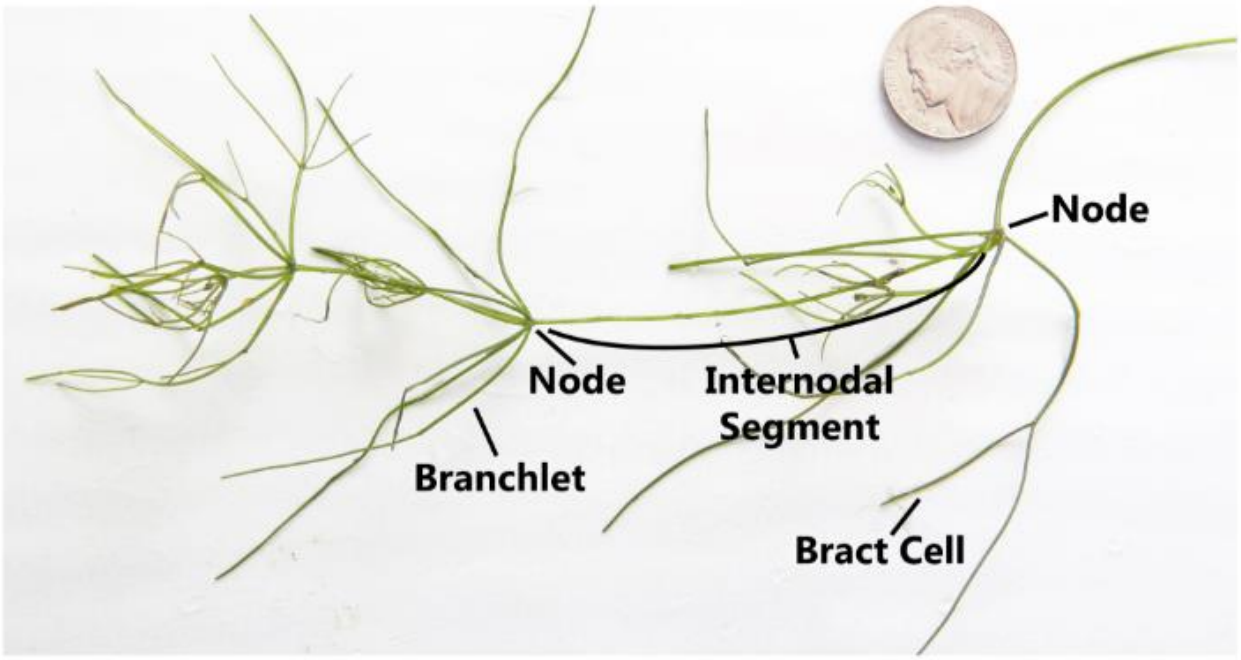


**Fig. 2.1** Starry stonewort *Nitellopsis obtusa* segment of thallus containing internodal cells and connective multicellular nodes with branchlets and bract cells (*credits: https://starrystonewort.org/facts/)*

*Nitellopsis obtusa* is a multicellular organism that grows up to 30 cm in length and has a cylindrical or slightly flattened stem (thallus). The plant produces whorls of branches at regular intervals, with each branch bearing a single multicellular node. The intermodal segment between the nodes is a single cell (average length is 8-10 cm). The thallus is anchored in the soil with colorless rhizoids. Instead of nodes, large rhizoid cells are connected by star-shaped bulbils, which are full of starch grains (McCourt et al. 1999). *Nitellopsis obtusa* is found in slow-moving or stagnant freshwater or brackish habitats, such as ponds, lakes, and rivers, and is widely distributed throughout North America and Europe (Beilby and Casanova 2014).

The internodal cell structure of *Nitellopsis obtus*a is similar to that of other plant cells. These coenocytic cells are encased by a cellulose cell wall that shields the cell from the high pressure of its

internal turgor (5-7 atm) as well as external pressure. A significant portion of the cell's interior (up to 95%) is occupied by a large central vacuole that is surrounded by the membrane called tonoplast. The cytoplasm, which forms a streaming layer between tonoplast and the layer of chloroplasts near the plasma membrane is up to 10 μm thick and moves at a rate of up to 100 μm/s wich is a unique feature of Characeae (Beilby and Casanova 2014).

The plants for the experiment were collected from fresh-water lake Stanka (Lithuania, Vilnius district) in November 2022 (Lithuania, Vilnius district) using gaffed anchor from a depth of 4 -8 m and then maintained in glass aquariums at room temperature (20±1 °C) under daylight conditions (95±0,19 μmol $m^{-2}$ $s^{-1}$ ) of light/dark photo regime of 12/12 h in tap water.

Prior to the experiment internodal cells were cut from neighboring internodal cells near the node. Separated cells were kept overnight in APW (artificial pond water) under daylight conditions.

## 2.2 Microelectrode technique arrangement

Two pairs of intracellular glass microelectrodes were used for the registration of single cell electrical signaling in two separate sites of the cell (**Fig. 2.2**) further indicated as “apical“ and “basal“ sites. Intracellular glass microelectrodes were made from borosilicate glass capillaries (inner Ø 0.84 mm, World Precision Instruments) with a tip diameter of 1-3 μm (8 – 20 MΩ) and filled with 3 M KCl. The internodal cell was placed in the registration chamber filled with control solution and immobilized with vaseline, creating five separate solution pools. Intracellular glass microelectrodes were impaled using a PatchMan micromanipulator, and a LANG positioning system LSTEP. in two sites of the cell approximately 3 cm from each other while the cell was monitored using a binocular. The silver

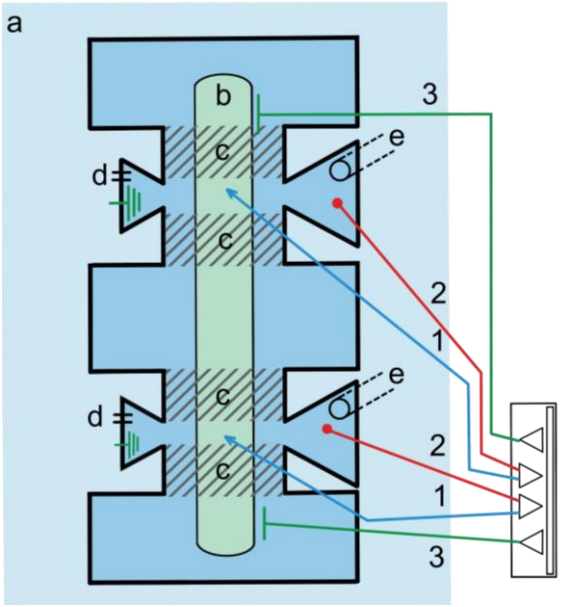


**Fig. 2.2** Microelectrode technique arrangement
a – plexiglass chamber
b – internodal cell
c – vaseline isolation of compartments (5 mm)
d,e - perfusion
1 – intracellular glass electrode
2 - reference glass electrode
3 – extracellular Ag/AgCl electrodes

reference electrodes were immersed in the vicinity of the cell in two different compartments near the intracellular electrode in each site. Direct current for stimulation was injected via extracellular silver wires in two separate circuits. For the computer I/O and data acquisition system, pCLAMP 10.2 data acquisition software from Molecular Devices, coupled with Digidata 1440A digitizer was utilized. Two amplifiers were used: the TEC-10CX two-pair voltage/current clamp amplifier, and .and the TEC-03X two-pair voltage/current clamp amplifier, from NPI Electronic. The signal gain was set at 10×, and the discretization frequency ranged from 100-1000 Hz. To perfuse the solution surrounding the cell in one of the compartments of the chamber, we employed a programmable perfusion system and vacuum waste kit (Scientifica PPS) at a flow rate of approximately 1 ml/min.

Characeaen cells are characterized by a large vacuole, which occupies approximately 95% of the cell volume (Mary J. Beilby and Casanova 2014). As a result, when a microelectrode is inserted into the cell, it typically penetrates the vacuole. Consequently, the potential difference measured between the microelectrode and the extracellular electrode reflects the combined potentials of both the plasmalemma and tonoplast membranes. A ramped current for electrical excitation was used. Unlike over-threshold current stimuli, this method allowed a precise measurement of the excitation threshold of membrane potential (Lapeikaitė 2020).

## 2.3 Experimental procedure

To prepare for electrophysiological experiments, the cell was placed in a borosilicate chamber and the central 5 mm compartment was isolated from adjacent compartments using vaseline. The chamber was then filled with APW, and the central compartment was subjected to perfusion. Two intracellular electrodes were inserted into the vacuole, approximately 3 cm apart. The cell was monitored under a binocular microscope (16×magnification). Experiments were conducted during the daytime, at room temperature (20±1 °C), and under constant illumination (95±0.19 µmol m-2 s-1). After the microelectrodes were inserted, at least 1 hour period of rest followed for the stabilization of the membrane potential. The APs were elicited by direct current that increased in a ramp rate of 0.02 µA/s from zero in current-clamp mode. Once the action potential threshold was reached, the stimulating current was manually stopped. Membrane resistance was evaluated by passing three hyperpolarizing current impulses of 1 s duration prior to each AP excitation. The electrophysiological procedures were conducted in the following order: three APs were evoked in the basal site with 5-minute interval between each excitation. After a 10-minute interval another three APs were evoked in apical site. After this protocol was completed, the control solution in the central compartment was replaced with the experimental solution of interest in each of the five solution pools, thus exposing the whole cell. This was achieved by retracting the control solution using a syringe and then applying the experimental solution using another syringe. The retraction and application steps were repeated three times to ensure proper replacement of the solutions. After a 30-minute exposure the electrophysiological procedure was repeated in the same manner (**Fig. 2.3**). After the experiment, the distance between electrodes was measured using digital caliper.

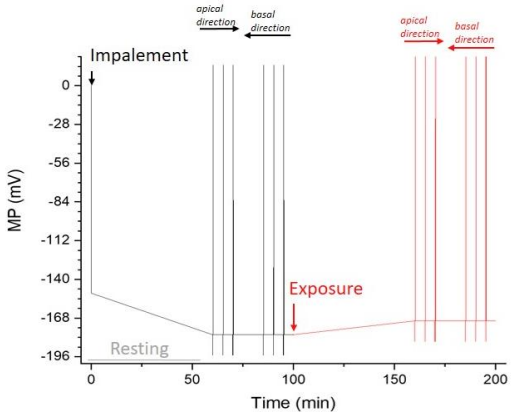


**Fig. 2.3** Protocol for the investigation of *Nitellopsis obtusa* internodal cell AP propagation velocity using two intracellular electrodes.
Red trace indicates exposure to a solution of interest. Arrows above indicate the recorded direction of AP propagation along the cell.

*Solutions:*

The control solution – APW' (0,1 mM KCl, 1,0 mM NaCl, 0,1 mM $CaCl_2$, 1 mM HCl and 3 mM tris(hydroxymethyl)aminomethane (TRIS), adjusted to a pH 7,2 by 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)).

The experimental solution – APW+ Glu-Cl (0,1 mM KCl, 1,0 mM NaCl, 0,1 mM $CaCl_2$, 1 mM GluCl and 3 mM TRIS, adjusted to a pH 7,2 by HEPES).

## 2.4 Electrophysiological parameters

Electrophysiological parameters were evaluated in CC mode (**Fig. 2.4**). All investigated AP parameters and criteria for them are listed in table 2.1.

**Table 2.1** List of investigated AP parameters in the intermodal cell of *Nitellopsis obtusa* using two intracellular electrodes

| Parameter | Unit | Evaluation method |
|---|---|---|
| Resting membrane potential (MP) | mV | was evaluated prior to AP excitation, also before and after exposure to presented compounds. |
| Membrane resistance (MR) | Ω/m2 | was evaluated by passing several hyperpolarizing current impulses of 1 s duration prior to AP excitation, also before and after 30 min exposure to presented compounds. Formula used for the calculation of MR: $R= \Delta U\ I\ \pi d l$, where ΔU - MP difference (V), I - applied current (A), *l* - cell length in central compartment (0,005 m); d - cell diameter (m). |
| AP excitation threshold ($E_{th}$) | mV | determined as the MP, when the depolarization rate exceeded 60 mV/s. |
| AP peak | mV | the maximum voltage reached during the depolarization phase |
| AP amplitude | mV | determined from $E_{th}$ to the AP peak potential |
| $\Delta U_{th}$ | mV | the difference between MP and $E_{th}$ |
| Repolarization duration (RD) | s | was evaluated as time span of 90 mV repolarization from peak potential |
| Peak to peak time difference (P-P) | s | the time interval between the peak of an electrically elicited AP and the peak of a transmitted AP |
| AP propagation velocity ($V_{prop}$) | cm/s | was calculated using the following formula: $V_{prop}= l/$ P-P, |

| | | |
|---|---|---|
| | | where *l* - distance between the electrodes (cm); P-P - peak to peak time difference (s). |

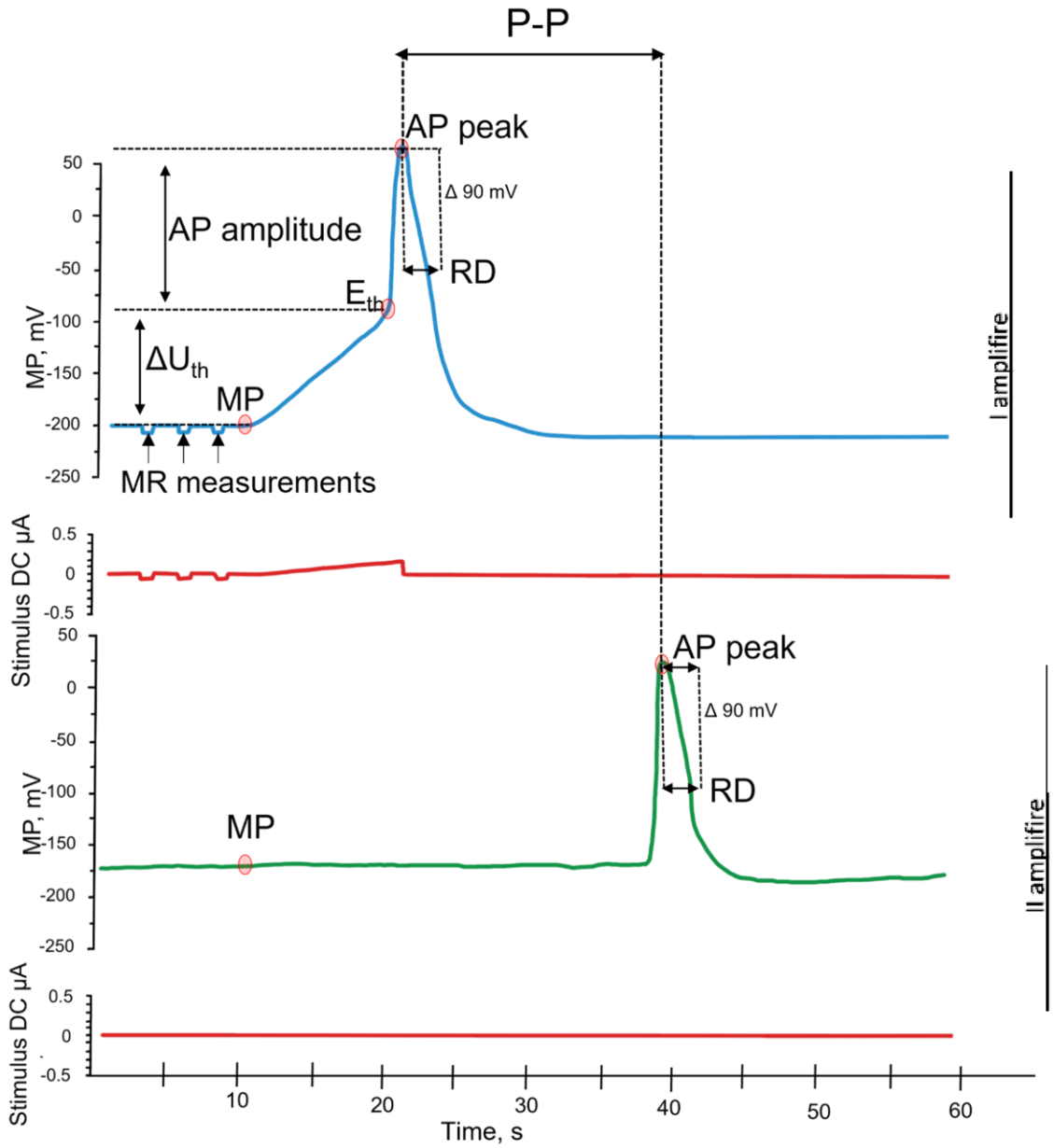


**Fig 2.4** Example of DC elicited AP (blue), propagated AP (green) and evaluated parameters in CC mode. $E_{th}$ - MP potential with depolarization exceeding 60 mV/s. AP amplitude – the MP interval from $E_{th}$ to the peak potential. $\Delta U_{th}$– the MP interval from $E_{th}$ to the peak potential. Repolarization duration (RD) – the time span of 90 mV repolarization from the peak. MR (membrane resistance) measurements – rectangular hyperpolarizing DC impulses. Peak to peak time difference (P-P) – the time interval between the peak of an electrically elicited AP and the peak of a transmitted AP (if the distance between electrodes is about 3 cm, then usual P-P value is about two seconds.

## 2.5 Statistics

To account for inherent variations in electrical signaling patterns, such as MP, $E_{th}$, AP amplitude, each parameter's value under the influence of glutamate was compared to its value in the control solution of the same cell. Electrophysiological parameters described in sec. 2.5 were measured for each of the six APs elicited in control solution and then compared with corresponding APs elicited after the exposure to glutamate. For instance, the velocity of the first AP in the apical direction (control condition: APW) was compared to the velocity of the first AP in the apical direction (experimental condition: 1 mM Glu), and so on for the second and third set of excitation. These measurements were repeated for each individual cells (n=15), and the results are presented as mean values ± standard error (SE), unless otherwise specified. The Shapiro-Wilk test was utilized to confirm the normality of samples, and a two-tailed paired Student's t-test was used to test differences within individual cells. Differences with $p<0.05$ were considered significant, denoted by asterisks in the results section. Correlations between parameters were assessed using the Pearson correlation coefficient for normally distributed samples and the Spearman's correlation coefficient for non-normally distributed samples. Data analysis was performed using the following software:

- *Clampfit* (Molecular Devices Corporation),
- *Excel* (Microsoft Office Corporation),
- *OriginPro* (OriginLab Corporation).

# 3. RESULTS AND DISCUSSION

## 3.1 Electrophysiological parameters in control solution

*Temporal control experiments*

Temporal control experiments conducted by Lapeikaitė (2020) confirmed electrophysiological parameters (MP, $E_{th}$. AP peak, AP Amplitude, RD and MR) of *Nitellopsis obtusa* intermodal cell to be stable during the 5,5 h experiment when measured in 30 min. intervals. Therefore, it was concluded that the resting periods established between measurement sets (30 minutes) were sufficient for stabilizing the cell state. Hence, it can be inferred that *Nitellopsis obtusa* does not demonstrate any intrinsic circadian drift of electrophysiological parameters, at least up to 8 hours during the daytime. Given that temporal control experiments have been previously conducted and confirmed the stability of electrophysiological parameters of *N. obtusa*, additional temporal control experiments for the investigated propagation velocity were not performed in this study.

*Control solutions*

APW solution was used as a control in all experiments. To establish proper control conditions (ion concentrations) for this investigation, APW was supplemented with an additional 1 mM of HCl (as opposed to 1 mM GluCl in the experimental condition). The prior studies(Lapeikaitė 2020) have shown that 0.5-1 hour exposure of *N. obtusa* to 1 mM HCl (7.2 pH) did not alter the membrane resting potential or resistance but slightly increased the AP amplitude (by 6,7±1,6 mV from 125±4 mV to -132±4 mV (p=0,01)). Based on the information provided, it can be concluded that APW supplementation with additional chloride is suitable control solution.

*Electrophysiological parameters*

Described experiments were performed with 15 cells. Due to the inherent variability in cellular parameters, a specific set of criteria was applied to select cells for further analysis. Firstly, cells were included if the resting potential was more negative than 100 mV and the difference between resting potential measurements recorded from two sites was no greater than 40 mV. Secondly, only cells that propagated all induced APs throughout the recording were chosen. Additionally, cells were considered if their resting potential in the control solution remained stable between the first and sixth action potentials, meaning that the cells did not enter another state of resting plasma membrane. Lastly, cells were selected if their action potential amplitude exceeded 90 mV.

This criterion aimed to identify cells (n=6) that met predetermined requirements or exhibited specific characteristics relevant to the study. Evaluated mean electrophysiological parameters' values

in control conditions of selected set of cells are presented in table 3.1. All parameters' followed a normal distribution except RD, MR, $V_{prop}$.

**Table 3.1** Electrophysiological parameters of *Nitellopsis obtusa* intermodal cell in control solution (APW) of all investigated cells in basal and apical sites, mean ± standard deviation, n=6.

| Parameter | Mean ± SD (apical site) | Mean ± SD (basal site) |
|---|---|---|
| RP (mV) | -179±30 | -173±33 |
| MR (Ω/m2) | 19±6 | 14±5 |
| $E_{th}$ (mV) | -95±15 | -94±12 |
| $\Delta U_{th}$ (mV) | 84±30 | 78±26 |
| AP peak (mV) | 20±6 | 20±5 |
| AP amplitude (mV) | 115±16 | 114±13 |
| RD (s) | 7±3 | 9±5 |
| | basal direction | apical direction |
| $V_{prop}$ (cm/s) | 1.36±0.6 | 1.7±0.87 |

In previous investigations (Lapeikaitė 2020), independent parameters of electrically induced APs exhibited no correlation between themselves. Neither $E_{th}$, AP peak, AP amplitude, nor repolarization duration correlated with RP, or with each other, supporting the idea that RP variability has no effect on excitation parameters (if the cell is in a constant state regarding RP) and the investigated AP parameters may be modulated independently.

*Propagation velocity*

First, the velocity values obtained in control condition (n=36: 18 AP in apical direction and 18 AP in basal direction) were examined. The assessed velocity vary from 0.47 cm/s to 3.56 cm/s (**Fig. 3.1**) and the mean velocity value is 1.54±0.93 cm/s with median of 1.2 cm/s.

The velocities of each AP propagating in apical versus basal direction (n=18, each direction) were examined. Results indicate that there is significant

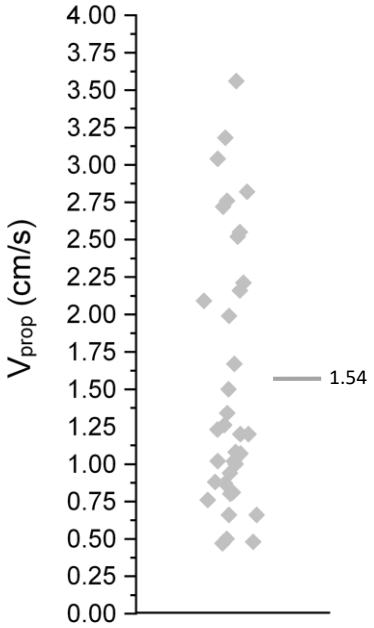


**Fig. 3.1** AP propagation velocities ($V_{prop}$) along the intermodal cell of *Nitellopsis obtusa* in both apical and basal direction (n=36) in control conditions (APW); grey diamonds represent AP propagation velocity value of each AP; solid grey line represents mean $V_{prop}$ value

differences (p=0.005) in the propagation velocities of APs between these directions. APs exhibited a higher propagation velocity (0.34 cm/s, 22±6%) to apical direction (1.7±0.8 cm/s) (propagation from the basal site of elicitation to the apical recording site) then in basal one (1.36±0.6 cm/s) **(Fig. 3.2**).

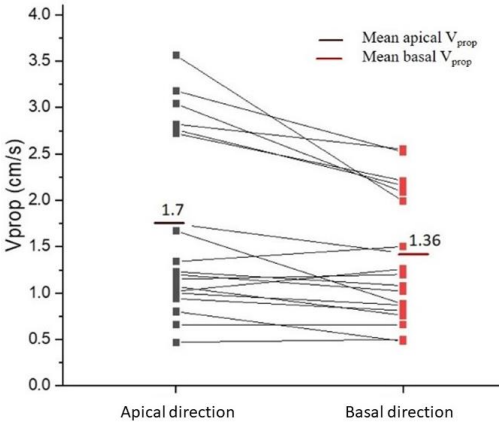


**Fig. 3.2** AP propagation velocities in apical versus basal directions along the internodal cell of *Nitellopsis obtusa* in control solution (APW), n=6 (individual cells) n=18 (analyzed APs).

Potential correlations between the $V_{prop}$ and other electrophysiological parameters (RP, $E_{th}$, RD, AP amplitude) were further investigated in control solution. However, the correlation analysis did not yield statistically significant results ($p<0.05$). Although, the correlation between $V_{prop}$ and MR was found to be significant, indicating that a higher membrane resistance allows for more efficient and rapid conduction of the action potential along the membrane (**Fig. 3.3-3.4**).

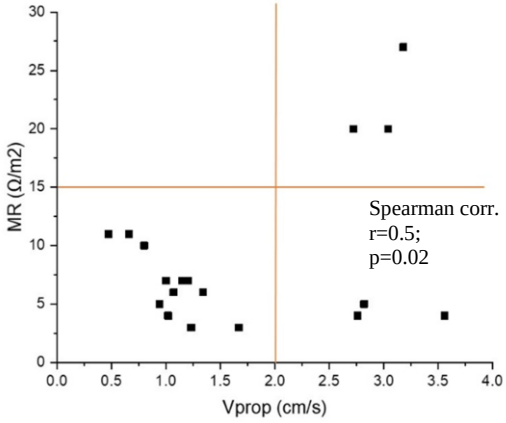


**Fig. 3.3** Correlation between AP propagation velocity ($V_{prop}$) in apical direction and the membrane resistance (MR) of each AP in control solution (APW), n=6 (individual cells) n=18 (APs).

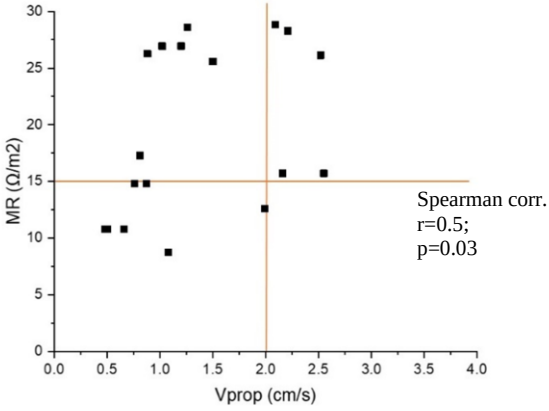


**Fig. 3.4** Correlation between AP propagation velocity ($V_{prop}$) in basal direction $V_{prop}$ and membrane resistance (MR) of each AP in control solution (APW), n=6 (individual cells) n=18 (APs).

A moderate positive correlation has been discovered between $\Delta U_{th}$ (the difference between MP and Eth) and the propagation velocity indicating that as the cells' MP is closer to the threshold required for excitation, this heightened excitability allows for more efficient and rapid propagation of APs (**Fig. 3.5**).

Summarizing, the results obtained in standard conditions indicated that the velocity values of electrically induced APs along the intermodal cell of *Nitellopsis obtusa* range from 0.47 cm/s to 3.56 cm/s, with a mean velocity of 1.54±0.93 cm/s. Significant differences were found between the direction of propagation, with higher velocities observed in the apical direction compared to the basal direction (22±6%). The only parameter directly correlating with the propagation velocity was $\Delta U_{th}$. Indicating that such AP parameters as RP, $E_{th}$, RD and AP amplitude not directly correspond to the velocity of AP.

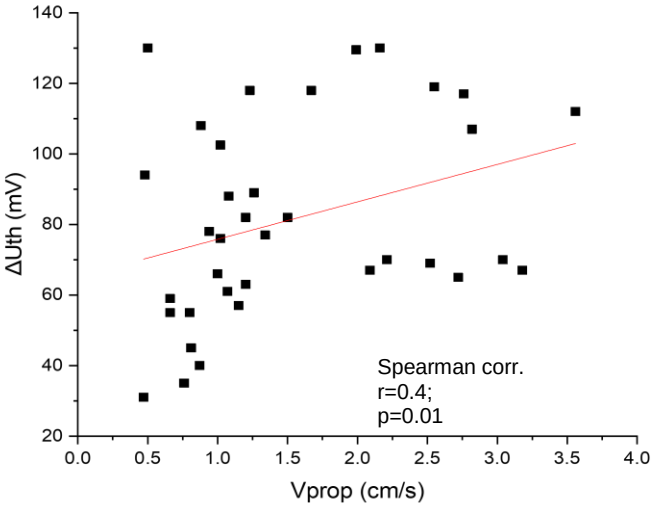


**Fig. 3.5** Correlation between AP propagation velocity ($V_{prop}$) and the difference between MP and $E_{th}$ ($\Delta U_{th}$) of each AP in control solution (APW), in both directions, n=6 (individual cells) n=36 (APs).

## 3.2 Effect of L-glutamate (natural GLR agonists) on excitation parameters and propagation velocity

The AP parameters of *Nitellopsis obtusa* internodal cell were investigated in experimental conditions and then compared to the values obtained in control solution in each cell. This was done after exposing the whole cell surface for 30 minutes to a buffered solutions containing 1mM of natural GLR agonists (L-glutamate). The AP parameters were studied using CC mode as described in section 2.3. The identical protocols in both control solutions and during/after exposure were followed. Since one of the primary plant cell responses to AA is membrane depolarization, RP during the exposure was monitored. In previous studies on *N. obtusa* (Lapeikaitė 2020), it was described that 1 mM Glu slightly depolarizes RP, prolongs the AP repolarization duration (RD) and hyperpolarizes the $E_{th}$, but doesn't alter AP peak values, thus the AP amplitude thus is increased by 11%. The membrane resistance was not significantly altered by Glu. In these studies, only a portion of the cell surface (0.5 cm) was exposed to Glu solution.

Our investigation is in coherence with the previous findings. After exposure to glutamate we observed slightly depolarized membrane resting potential in both apical (depolarized by 5.2%, from -

173 mV to -164 mV; p= 0.038) and basal regions (depolarized by 9.2%, from -179 mV to -172 mV; p= 0.064). $E_{th}$ after exposure was also hyperpolarized in both apical (from -95 mV to -118 mV, 25.5%, p<0,001) and basal regions (from -94 mV to -98 mV by 4.25%, p=0.03). AP peak values did not differ (19±7mV vs 18±6 mV in apical side; 20±7 mV vs 22±6 mV in basal side). These electrophysiological parameters measured in standard conditions and after exposure to Glu are presented in **Fig. 3.6**.

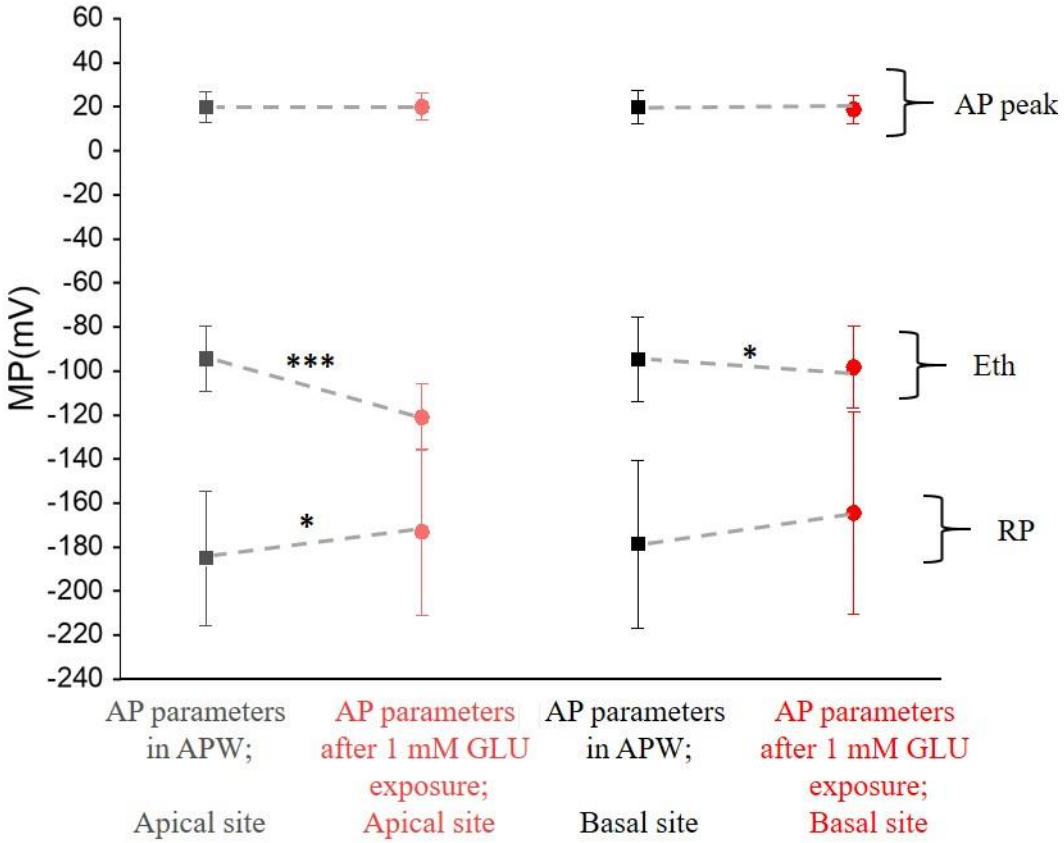


**Fig. 3.6** Average $E_{th}$, AP peak and RP values in control solution (APW) and after the exposure to 1mM Glu, n=6 individual cells.

Given the hyperpolarized $E_{th}$ and unchanged AP peaks, APs after Glu application featured heightened amplitudes (average increase in amplitude is 15±3mV; 13% p<<0,001). Upon exposure to Glu, a noticeable increase in the repolarization duration was observed (8±5 s in control solution vs 11±9s in experimental solution; p=0.04, both directions, n=36). Membrane resistance was not altered (13.8±6 $\Omega m^2$ in control solution vs 13.3±6 $\Omega m^2$ after the exposure in apical side; 19±7 $\Omega m^2$ vs 17±7 $\Omega m^2$ in basal side). Given depolarized MP and hyperpolarized $E_{th}$, it was expectable to observe

decreased $\Delta U_{th}$ after the exposure to Glu. $\Delta U_{th}$ was decreased from 78 mV to 66 mV (p= 0.006) on apical site and from 84 mV to 64 mV (p= <<0,001) on basal site (**Fig. 3.7**).

Summarizing data, as expected after the exposure of *N. obtusa* intermodal cell to Glu, electrically induced APs are prolonged, exhibiting heightened amplitudes and hyperpolarized $E_{th}$. $\Delta U_{th}$ after the exposure was significantly decreased, given depolarized MP and hyperpolarized $E_{th}$. The exposure of the entire cell surface (in contrast to 0.5 cm exposed surface in previous studies) to glutamate did not result in increased alterations of electrophysiological parameters (for example 13% increase in amplitude when whole cell is exposed in comparison to 11% when portion of cell in exposed).

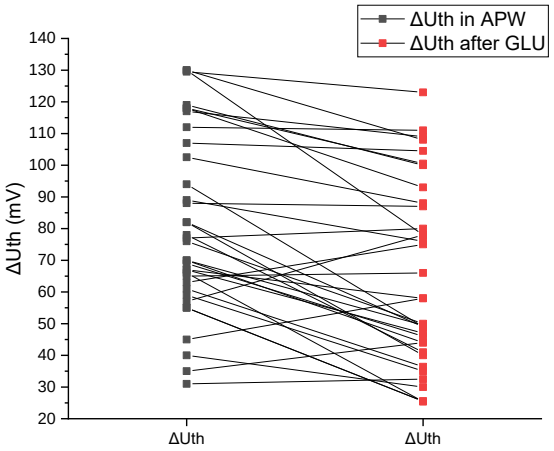


**Fig 3.7** The comparison of $\Delta U_{th}$ (the difference between MP and $E_{th}$ ) values in control solution (APW) and after exposure to 1 mM Glu, combined set of APs (both apical and basal), n=6 (individual cells) n=36 (APs).

*Effect of L-glutamate on propagation velocity*

To investigate the effect of L-glutamate on AP propagation velocity the velocity values of each of the 36 APs (18 APs in apical direction and 18 APs in basal direction) were analyzed in control solution and then compared to their counterpart after the exposure to 1mM Glu.

The combined set of AP $V_{prop}$ in both apical and basal direction (n=36, n=18 in each direction of 6 individual cells) after the exposure to 1mM Glu was analyzed in comparison to the similar set of values obtained in control condition. The velocity values obtained in the experimental condition show a similar distribution pattern to the values obtained in the control condition. The individual values range from 0.76 cm/s to 3.37 cm/s. (**Fig. 3.8**). The mean velocity value is 1.75±0.77 cm/s. The median velocity value is 1.54 cm/s.

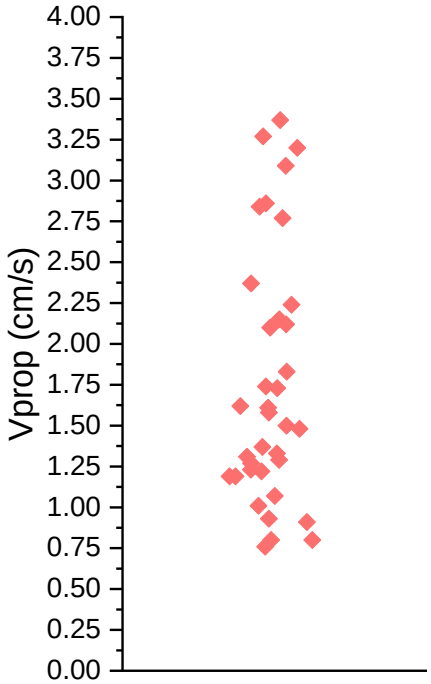


**Fig. 3.8** The combined set of AP propagation velocities ($V_{prop}$) in both apical and basal direction after the exposure to 1mM Glu. n=6 (individual cells), n=36 (APs).

The velocity values of corresponding APs were compared. Out of 36 APs, 2 showed no change, 5 exhibited a decrease (from 0.9 cm/s to 0.04 cm/s), and 29 demonstrated a significant (p=0.002) increase after exposure to glutamate (**Fig. 3.9**). The average increase in AP propagation velocity is 0.39 cm/s. It is also worth noting that these individual increases in AP velocity exhibit a considerable

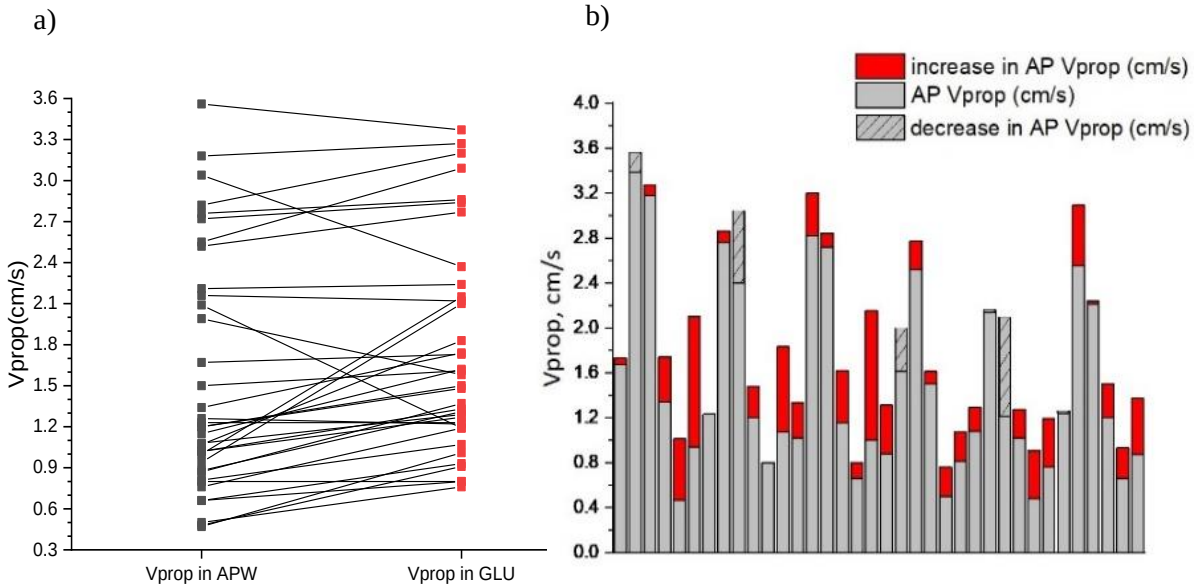


**Fig. 3.9** The comparison of AP propagation velocity ($V_{prop}$) in standard conditions (APW) and after the exposure to1 mM Glu, combined set of both apical and basal directions. n=6 (individual cells) , n=36 (APs)**a)** $V_{prop}$ in control solution (APW) represented by grey color versus in experimental solution (1 mM Glu) represented by red color
**b)** the grey columns represent the initial values of $V_{prop}$ in control conditions, the red columns above the grey one represent the increase in $V_{prop}$ after the exposure to 1 mM Glu, the sparse pattern overlapping with the grey columns represent the decrease in $V_{prop}$ after the exposure to 1 mM Glu.

amount of variability, with some individual APs experiencing only a slight increase (minimum increase 0.03 cm/s) while others experience larger increases (maximum increase 1.16 cm/s).

The statistical analysis revealed a significant negative correlation between AP $V_{prop}$ and the corresponding change in $V_{prop}$ after exposure to 1mM Glu (**Fig. 3.10**). This means that if the initial value of AP $V_{prop}$ is high, the magnitude of change in AP $V_{prop}$ following exposure to 1mM Glu will be smaller compared to if the initial value is low.

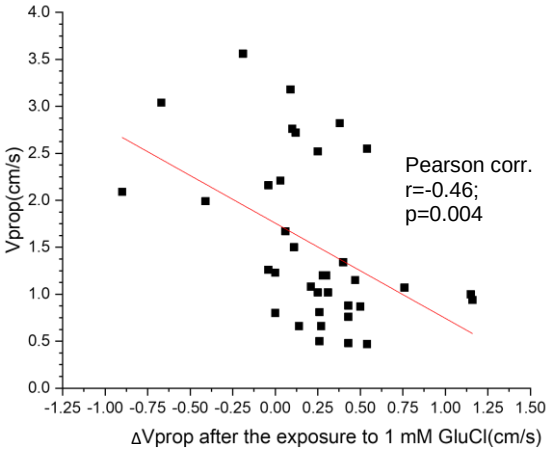


**Fig. 3.10** Correlation between AP propagation velocity ($V_{prop}$) and corresponding change in $V_{prop}$ ($\Delta V_{prop}$) after the exposure to1 mM Glu, combined set of both apical and basal AP (n=36) from 6 cells

Next step after analyzing the combined set (apical and basal directions combined together) of $V_{prop}$ in the control condition versus experimental condition was to specifically compare the apical direction of propagation in the control condition with the apical direction in the experimental condition, and likewise for the basal direction.

Externally applied glutamate increases propagation velocity of electrically induced APs in both apical (from 1.7 cm/s to 1.98 cm/s; p= 0.01) (32±10%) and basal (from 1.36 cm/s to 1.52 cm/s; p= 0.03) (23±7%) directions (**Fig. 3.11**). The mean increase of $V_{prop}$ in apical direction (0.28 cm/s) was not significantly higher than in basal direction (0.16 cm/s), p= 0.07.

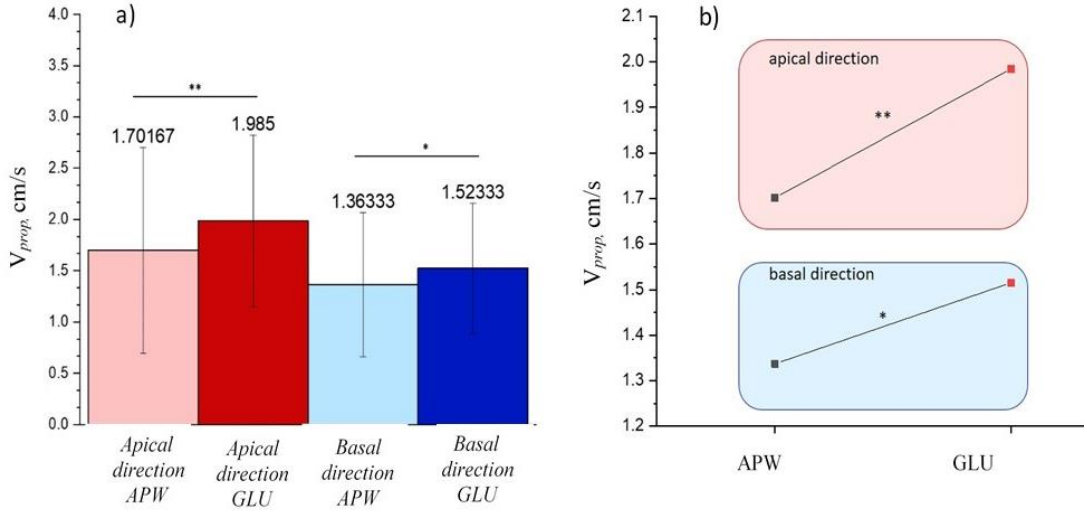


**Fig. 3.11** Mean values of AP propagation velocity ($V_{prop}$) along the intermodal cell of *Nitellopsis obtusa* in standard conditions (APW) and after the exposure to1 mM Glu. Asterisks indicate significant difference, n=18 AP (from 6 cells) in each column.

Summarizing, the exposure to 1mM Glu resulted in a significant increase in the propagation velocity of APs in both apical and basal directions, with 29 out of 36 APs showing increased velocities, ranging from 0.04 cm/s increase to 1.16 cm/s increase. There was a significant negative correlation between the initial AP velocity and the magnitude of change in velocity after glutamate exposure, indicating that higher initial velocities resulted in smaller changes. Additionally, the mean increase in AP propagation velocity was 0.28 cm/s in the apical direction and 0.16 cm/s in the basal direction, although the difference was not statistically significant.

*Glutamate enhanced electrically induced signaling (in plant cells lacking AP propagation in control conditions)*

In a sample of 15 plant cells subjected to depolarizing stimuli, a subset of cells (33.3%; n=5) did not exhibit AP propagation in control conditions, meaning that in one recording site elicited AP was not propagated to the other recording site. Membrane RP of these cells ranged from -142 mV to

**Table 3.2** Change in the electrophysiological parameters of *Nitellopsis obtusa* intermodal cells lacking the ability to propagate AP in control solution (APW) of all investigated cells in basal and apical direction, mean ± standard deviation, n=6.

| ***Cell*** | ***Ability to propagate AP before the exposure*** | ***Ability to propagate AP after the exposure*** | ***Change in MP after the exposure, mV*** | ***Change in $E_{th}$ after the exposure, mV*** | ***Change in ΔUth after the exposure, mV*** |
|---|---|---|---|---|---|
| 1 | Passed GP | Passed AP | ↑ +19.5 | ↑ +9 | ↓ -10 |
| 2 | No passing of AP | Passed AP | ↓ -17.5 | ↓ -1 | ↑ +17 |
| 3 | No passing of AP | Passed AP | ↓ -4 | ↓ -23.5 | ↓ -21.5 |
| 4 | No passing of AP | Passed AP | ↓ -1.6 | ↓ -23.5 | ↓ -1.5 |
| 5 | No passing of AP | Passed GP | ↑ +12.5 | ↓ -1 | ↓ -14 |

-232 mV (mean -185±30 mV). The APs induced on the excitation site were of normal physiological parameters with slightly depolarized $E_{th}$ (-84 mV) and peak value (13 mV), although those electrically induced AP either did not propagate or propagated as gradual potentials (GP). 1st plant cell was the only one to pass the gradual potential in the control solution, while the remaining 4 did not pass any potentials. Overview of cells lacking AP propagation is presented in **Table 3.2**.

Following exposure to 1mM Glu, this subset of cells displayed a significant increase in their signaling ability. After the exposure, four cells started passing action potentials of normal amplitude, and the 5th cell passed gradual potentials, demonstrating a glutamate-induced enhancement of depolarization-induced signaling (mean $V_{prop}$=0.88 cm/s). Although, glutamate had a positive effect on the electrical signaling in these specific cells, the speed of propagating of these APs still exhibits a lower velocity compared to the average Vprop after the exposure to glutamate (1.4 cm/s).

1st cell (passed GP in control; AP after the exposure) and the 5th cell (inactive in control; passed GP after the exposure) experienced considerable depolarization of membrane potential (33.5%). 2nd, 3rd and 4th cells (inactive in control; passed AP after the exposure), in contrary, displayed hyperpolarization of membrane potential (15%). $V_{prop}$ velocity values positively correlated with the change in $E_{th}$ (**Fig. 3.12**).

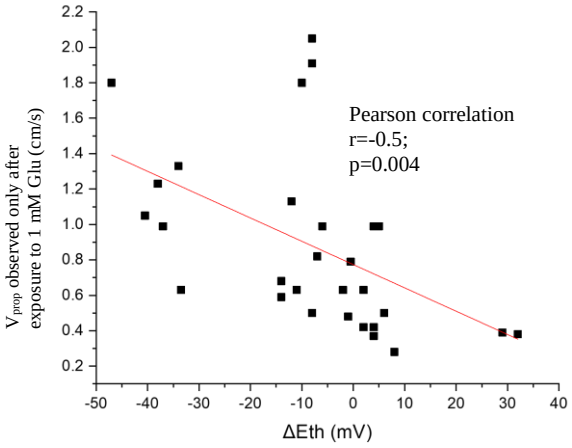


**Fig. 3.12** Correlation between $V_{prop}$ after the exposure and $\Delta E_{th}$ of each AP in both directions, n=30.

Summarizing this part, there are evidence that exposure to 1 mM Glu enhance electrically induced signaling in cells lacking the ability to propagate AP in control conditions. The effect of Glu on other electrophysiological parameters of AP in this subset of cell was standard (prolonged RD, decreased $\Delta U_{th}$), except for the $E_{th}$ (was not significantly hyperpolarized) and MP (was hyperpolarized in 2 cells and depolarized in 3 cells).

## 3.3 The correlation between glu-induced changes of AP's parameters and AP propagation velocity

As already stated, after the exposure of *N. obtusa* intermodal cells to Glu, the electrically induced APs exhibited prolonged durations, increased amplitudes, hyperpolarized thresholds and decreased $\Delta U_{th}$ in both apical and basal site of the cells. In this section, the results of the effect of the potential correlations between the mentioned alterations in AP parameters ($\Delta$) and the change in propagation velocity is presented ($\Delta V_{prop}$). The combined set of both apical and basal APs was used, due to the fact that Glu affected $V_{prop}$ in different directions in a compatible manner, as stated in section 3.2.

The correlations observed between the alterations in AP parameters and the change in propagation velocity were not significant (n/s). Although, a moderate negative correlation found between prolonged duration of APs and the change in velocity (Pearson correlation r=-0.32; p=0.07) was near significant. (**Fig. 3.13**).

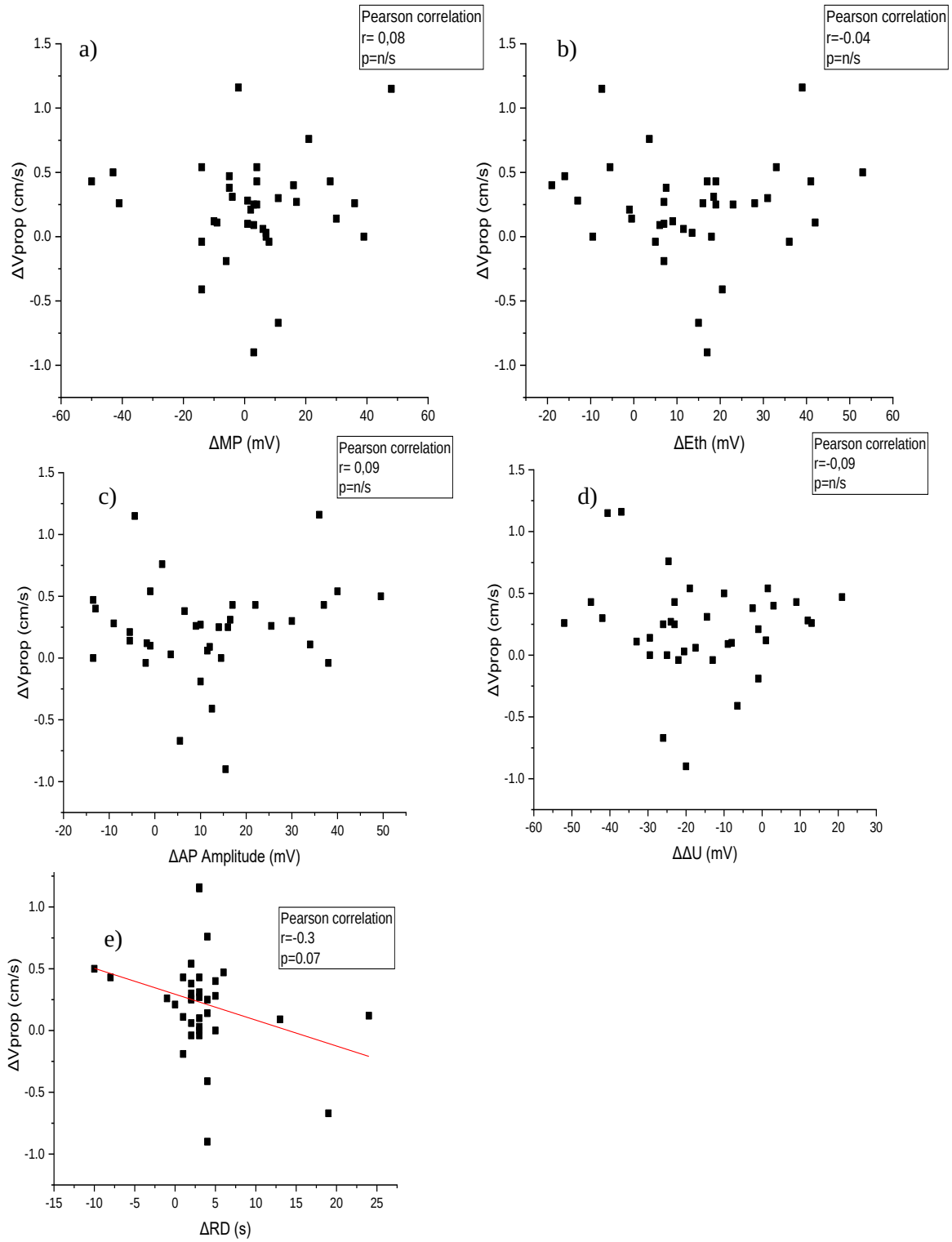


**Fig. 3.13** Correlations between Glu induced AP alterations and changes of AP propagation velocity: **a)** between the change of AP propagation velocity ($\Delta V_{prop}$) and corresponding change resting membrane potential (ΔMP) of each AP in both directions, n=36; **b)** $\Delta V_{prop}$ and corresponding $\Delta E_{th}$ of each AP in both directions, n=36; **c)** $\Delta V_{prop}$ and corresponding ΔAP amplitude of each AP in both directions, n=36; **d)** $\Delta V_{prop}$ and corresponding $\Delta U_{th}$ of each AP in both directions, n=36; **e)** $\Delta V_{prop}$ and corresponding ΔRD of each AP in both directions, cells n=36.

## 3.4 Discussion

*Velocities of AP in apical and basal directions in control condition*

A significant difference between APs' propagation velocities along the apical and basal directions was observed. These results suggest that the propagation velocity of APs is direction-dependent in *N.obtusa* intermodal call and may be influenced by factors such as the local thallus structure and the distribution of ion channels along the cell tissues. A similar finding regarding the direction-dependent propagation velocities of APs was reported for *Drosera rotundifolia*. AP transmission velocities were observed to be 0.43 cm/s in the basipetal direction (basal) and 0.99 cm/s in the acropetal direction (apical) (Williams and Spanswick 1976). Another study on *Dionea* trap recorded that the transmission velocity of the action potential is higher in the direction parallel to the vascular bundles than in the perpendicular direction (Pickard 1973). Studies on *Helianthus annuus* (sunflower) (Zawadzki, Dziubińska, and Davies 1995) and *Salix viminalis* (Willow Trees) (Fromm And Spanswick 1993) did not reveal any differences between the basipetal and acropetal directions. A contrary result comes from a study on slow fluctuations of membrane potential in various plant species, such as *Arum, Croton, Ficus, Inula, Rumex, Saxifraga, Viburnum, Phaseolus,* and *Rhynchosia*. It was found that the rates of slow fluctuations, elicited by mechanical stimulation, were generally higher in the basipetal direction compared to the acropetal direction. The observed fluctuations were at least in part variation potentials (Pickard 1973).

These results collectively support the idea that AP propagation velocity is dependent on the direction of transmission along the axes of plant thallus, also tissue structure

*Correlation between $V_{prop}$ and other electrophysiological parameters in control condition*

According to mathematical model of AP propagation in higher plants (Pyatygin 2006), depolarized resting MP, a hyperpolarized $E_{th}$ and reduced difference between MP and $E_{th}$ ($\Delta U_{th}$) also a higher MR can all contribute to a faster APs with higher amplitudes in plant cells. A more negative resting potential can increase the probability of opening voltage-gated calcium channels, leading to a faster and stronger depolarization phase of the action potential. This is because a more negative resting potential creates a larger electrochemical gradient for calcium ions to flow into the cytoplasm during depolarization. A lower excitation threshold can also contribute to a faster and stronger action potential by allowing the AP to be triggered with a smaller depolarization stimulus. This is because a lower excitation threshold implies that the balance of depolarizing and hyperpolarizing currents is shifted towards depolarization, making it easier to reach the threshold for action potential initiation

(Sukhov and Vodeneev 2009). While mentioned relations between parameters and AP velocity are based on mathematical model and data, our investigation in *Nitellopsis obtusa* did not reveal any significant correlations between these parameters (resting potential, excitation threshold, repolarization duration, AP amplitude). Although, $\Delta U_{th}$ (the difference between MP and $E_{th}$) and $V_{prop}$ positively correlated (**Fig. 3.5**), controversy to the negative correlation predicted by mathematical model. The correlation between $V_{prop}$ and MR was found to be significant and positive in control solution (**Fig 3.3-3.4**). A higher MR can contribute to a faster AP by reducing the leak of ions across the plasma membrane and minimizing the attenuation of the signal. This is because a higher membrane resistance implies that the plasma membrane is more resistant to ion flow, which helps to maintain the depolarization and allows the action potential to propagate efficiently along the cell membrane.

*Glutamate-induced increase in the propagation velocity*

Presented data indicated that in the internodal cell of *Nitellopsis obtusa* the externally applied glutamate not only alters the AP characteristics but also simultaneously increases the propagation velocity of electrically induced APs in both apical and basal directions (**Fig 3.9**).

It has been shown that in *Arabidopsis*, glutamate activate GLR ion channels that allow the flow of cations (specifically $Ca^{2+}$) into the cell (Weiland et al. 2015), also activation of GLR seems to increase the amplitude of AP due to hyperpolarized threshold. These effects are contributed to calcium signaling (Weiland et al. 2015).

In *Nitellopsis* glutamate-induced increase in AP propagation velocity in both directions along the cell could also be due to altered calcium signaling, although Characeae do not possess direct homologs fot *At*GLR.

It is plausible that hyperpolarized Eth and increases amplitude upon Glu in Characeae is also due to altered calcium signaling in the cytoplasm.

Slight MP depolarization was also observed in *Nitellopsis*, no significant correlation between MP and $V_{prop}$ was found. Interestingly, that the cells lacking the ability to propagate AP in control conditions indeed experienced significant depolarization of MP after the exposure to glutamate and they gained the ability to propagate AP, supporting the idea that glutamate indeed may play a role in modulating cellular excitability, thus facilitating more efficient AP propagation probably through calcium signaling. The lack of a significant correlation between MP and $V_{prop}$ in our study suggests that only depolarization of membrane potential is not sufficient for facilitating AP propagation. Other factors or mechanisms (such as the activation of specific channels or signaling pathways) may be involved in the enhanced AP propagation observed after the exposure to glutamate.

*Correlations between Glu induced alterations of AP and its propagation velocity*

In control condition $V_{prop}$ correlated with MR. Although, the exposure to Glu affected $V_{prop}$ but not the MR. The possible explanation for that could be that the increase in AP propagation velocity induced by glutamate may involve changes in the distribution or activation of specific ion channels that facilitate faster and more synchronized depolarization. However, these changes in ion channel activity may not directly affect the overall membrane resistance.

Although correlations between alterations in AP parameters and changes in propagation velocity were generally not significant, a moderate negative correlation was found between prolonged AP duration and the change in velocity. This suggests that APs with longer durations may experience smaller changes in propagation velocity after exposure to glutamate, indicating potential compensatory mechanisms in response to altered AP dynamics. One possible compensatory mechanism could be the activation of additional ion channels or modulation of existing ion channels in response to prolonged AP duration. This could serve to regulate the flow of ions and restore or maintain a certain level of membrane potential or depolarization rate necessary for efficient AP propagation. For example, the activation of potassium channels could enhance repolarization and shorten the duration of the AP, thus counteracting the prolongation caused by glutamate. By regulating the repolarization phase, these compensatory mechanisms may help maintain the overall efficiency and stability of AP propagation despite alterations in AP duration. Alternatively, since the correlation was moderate, it could imply that while the duration of APs may have some influence on the change in propagation velocity, additional factors may contribute to the observed alterations in AP parameters in *N. obtusa* intermodal cells exposed to Glu.

Additionally, a significant negative correlation between AP $V_{prop}$ and the corresponding change in $V_{prop}$ after exposure to 1mM Glu was found. This correlation suggests that the initial values of AP propagation velocity are an important determinant of the sensitivity of APs to the effects of Glu. One possible explanation for this correlation could be related to the concept of "ceiling effects" or saturation. It is possible that cells with higher initial propagation velocities have already reached a near-optimal state for AP propagation, and as a result, the potential for further enhancement in velocity is limited. In other words, there may be a maximum threshold or "ceiling" beyond which the AP propagation velocity cannot be significantly increased. Factors such as the distribution and activation of ion channels, changes in membrane properties, or other signaling pathways could contribute to the observed relationship between initial $V_{prop}$ and the change induced by glutamate.

*The function of glutamate-induced increase in AP propagation velocity*

Since the Glu can be both internal signal upon wounding (Seifi 2013) and environmental factor, the enhanced electrical signaling upon AA can lead to more efficient and rapid transmission of signals throughout the plant. Glutamate is known to be involved in plant responses to various environmental stimuli, including stress, light, and pathogens (Forde and Lea 2007). The increased AP propagation velocity triggered by glutamate could enhance the plant's ability to transmit and integrate signals related to these stimuli, enabling more efficient and timely responses to environmental changes.

Another feature of AP propagation is the involvement in the coordination of various physiological processes (growth, respiration (Pyatygin et al. 2008)). The increased efficiency of AP propagation induced by glutamate may contribute to the synchronized activation of different regions of the plant, allowing for coordinated physiological responses and adaptive behaviors. It is clear that in plant physiology Glutamate acts as a central hub for integrating multiple signaling pathways (Qiu et al. 2020), thus AP propagation velocity modulation as a response to glutamate may fine-tune the coordination and integration of different signaling pathways, enabling cross talk between various cellular processes and facilitating complex physiological responses

Taken all results into account it can be concluded that the APs propagation velocity in *N. obtusa* internodal cells is influenced by direction of propagation (higher in apical direction) within the cell and the presence of glutamate. Extracellular glutamate increases the APs propagation velocity in *N. obtusa* internodal cells, indicating its importance in modulating cellular excitability and facilitating efficient signal transmission.

*Possible directions for future research*

To further understand mechanisms underlying the enhanced AP propagation observed after glutamate exposure, future studies can focus on identifying and characterizing (expression patterns, distribution, and functional properties) the ion channels that are modulated by glutamate in in *N. obtusa*. Voltage clamp techniques can be a valuable tool in future investigation of the underlying mechanisms of AP propagation. By measuring and manipulating ion currents across the plasma membrane while applying glutamate, it would be possible to examine how glutamate modulates ion channel conductance, open probabilities, and voltage-dependent properties. Further research involving investigating of intracellular signaling cascades, second messenger systems, and the interactions between glutamate receptors and downstream effectors will contribute to understanding of the molecular mechanisms by which glutamate modulates plant cell electrical properties.

## CONCLUSIONS

1. In standard conditions, action potential propagation velocity along the intermodal cell of *Nitellopsis obtusa* is higher (22% ± 6%) to the apical direction compared to the basal one.

2. Externally applied glutamate increases propagation velocity of electrically induced action potentials in both apical (32% ± 10%) and basal (23% ± 7%) directions.

3. The glutamate-induced alterations in action potential parameters (hyperpolarized excitation threshold, heightened amplitude, decreased difference between membrane potential and excitation threshold) does not correlate with the change in propagation velocity.

## LIST OF PUBLICATIONS

**Conference Proceedings:**

1) **S. Blashchuk**, I. Lapeikaite, V. Pupkis, V. Kisnieriene Electrophysiological investigation of the effect of glutamate on the propagation of action potentials in a single plant cell. VII International Scientific Conference "Actual Issues in the Development of Biology and Ecology", Vinnytsia, Ukraine, November 16 - 17, 2022

2) **S. Blashchuk**, I. Lapeikaite, V. Pupkis, V. Kisnieriene Electrophysiological investigation of the effect of glutamate on the propagation of action potentials in a single plant cell. International Conference of Life Sciences “The COINS 2023”, Vilnius, Lithuania, April 24-26, 2023

VILNIUS UNIVERSITY

LIFE SCIENCES CENTER

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Master thesis

# ELECTROPHYSIOLOGICAL INVESTIGATION OF THE EFFECT OF GLUTAMATE ON THE PROPAGATION OF ACTION POTENTIALS IN A SINGLE PLANT CELL

## SUMMARY

Action potentials (APs) in plants are transient and propagating changes in the electrical potential across the cell membrane. Their propagation velocity depends on various electrophysiological parameters of the cell and the AP). In plants APs shape can be altered by various compounds and external factors. One of those compounds, amino acid glutamate, alter the electrophysiological parameters of AP (e.g. hyperpolarizes the AP excitation threshold, prolongs AP duration). The impact of these alterations upon glutamate on AP propagation velocity remain unknown.

This study aims to investigate the effect of glutamate (Glu) on AP propagation in a single plant cell. Objectives of the study are to determine AP propagation velocities along the cell of *Nitellopsis obtusa* in different directions under standard conditions and to investigate the effects of exogenous Glu on AP propagation velocity, also to assess whether Glu-induced alterations of AP parameters correspond to changes in AP propagation velocity.

Electrophysiological experiments were conducted using two intracellular microelectrodes impaled in two opposite sights of cell. APs were elicited by current in one sight of cell and later recorded in another (and *vise versa*). The effects of Glu was assessed by replacing the control solution with an experimental solution in a whole cell surface for 30 min.

This study demonstrates that AP propagation velocity in *N. obtusa* cells in control condition is dependent on the direction of propagation, with higher velocities observed in the apical direction (22± 6%). Exogenous glutamate enhances AP propagation velocity in both apical (32±10%) and basal (23±7%) directions. However, the specific alterations in AP parameters induced by Glu do not consistently correspond to changes in propagation velocity. Presented results indicate that glutamate signaling function encompass the electrical signaling propagation velocity alterations, also, although AP parameters and propagation velocity occur simultaneously they do not inherently correlate.

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