Lab Report

Electrophysiological characterization of a GluA4 gamma 2 tandem and investigation of first event amplitudes in single channel recordings

By: Lukas Born

Matriculation Number: 4003940

Date of submission: 30.09.2024

Keywords:

|  |
| --- |
| Scientific Topic (3-5 keywords):  transmembrane auxiliary AMPA proteins, AMPA subconductance levels, single channel recordings, xxx |
| Methods (3-5 keywords):  Macroscopic patch clamp recordings, single channel patch clamp recordings, xxxxx |

Please tick 2 fields at max.:

|  |  |
| --- | --- |
| ⌧ Cellular Neuroscience | 🞎 Clinical/Translational Neuroscience |
| ⌧ Molecular Neuroscience | 🞎 Cognitive Neuroscience |
| 🞎 Systems Neuroscience | 🞎 Computational Neuroscience |

Direct supervisor: Dr. Jenn Noonan

|  |  |
| --- | --- |
| Lab Leader:  Prof. Dr. Andrew Plested | Suggested Second Reviewer: |
|  |  |

**1 Introduction**

* 1. **AMPA significance, function and structure**

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are a type of ionotropic glutamate receptor (iGluR), which also include N-methyl-D-Aspartate (NMDA) and kainate receptors. During glutamatergic signaling, glutamate (Glu) gets released from the presynapse, diffuses across the synaptic cleft and binds to receptors. iGluRs undergo conformational rearrangement upon glutamate binding, which opens an ion pore, causing cation influx across the negatively polarized postsynaptic membrane, leading to depolarization (Collingridge & Lester, 1989; Dingledine et al., 1999; Meldrum, 2000; Traynelis et al., 2010, Aldrich et al., 1983; Auerbach, 2013; Plested, 2016). AMPARs are the fastest of the iGluRs, activating within 200-600 microseconds (µs) of Glu application, and almost completely desensitize within tens of milliseconds (ms) (Hestin 1990, Colquhoun 1992, Geiger 1995, silver 1992). This allows AMPARs to closely follow presynaptic input with the temporal accuracy necessary for precise high frequency signal transduction between neurons.

AMPARs are tightly regulated via subunit composition, phosphorylation or via interactions with other proteins (xxxxx). Alterations in localization, number and kinetics of AMPARs cause alterations in properties of synapses themselves, like long-term potentiation (LTP) or long-term depression (LTD), and are thus involved in several fundamental brain processes like acquisition and storage of memories (xxxxx). Conversely, their dysfunction is involved with several CNS pathologies (xxxxx)

AMPAs are composed as homo- or heterotetramers of four subunits, of which four subtypes exist: GluA1-4. Each subunit is composed of three domains: a transmembrane domain (TMD), a ligand-binding domain (LBD), and an amino-terminal domain (ATD) (Figure 1A). The function of the ATD remains unclear, as its deletion leaves causes no change in receptor function, but it has been proposed to play a role in receptor assembly and trafficking (Herguedas et al., 2013; Watson et al., 2017). The TMD is composed of four helices and contains the highly conserved nonselective cation channel (Bowie & Mayer, 1995; Kuner et al., 2003; Sobolevsky et al., 2003). The LBD is comprised of two segments, which form a clamshell shape, with a Glu binding site in between the two lobes of the clamshell (Armstrong & Gouaux, 2000; Stern-Bach et al., 1994). Upon binding glutamate, the clamshell closes and dimerizes with the LBD of another AMPA subunit, which pulls on the linkers between the LBD and the TMD, in turn opening the channel (Quiocho & Ledvina, 1996). Shortly after, the dimer between the ligand-bound LBD ruptures, and the channel closes again, causing desensitization (Mayer et al 2006) (Figure 1B).

Xxxx Just a quick sentence about the polyamine block of GluA2

* 1. **Transmembrane AMPA regulatory proteins**

AMPARs are regulated by binding to auxiliary subunits: transmembrane AMPAR regulatory proteins (TARPs) like stargazin, also known as GAMMA2, or TARP GAMMA8 (Bats et al., 2007, Payne 2008). TARPs not only anchor the receptor at the synapse but can also alter receptor dynamics and kinetics (Priel et al.2005, Coombs et al., 2017). Specifically, AMPARs in complex with GAMMA8 have been shown to produce a desensitization-resistant response after 10-25 Hz applications of glutamate, resulting in a distinct “pedestal” current (Plested and Carbone 2016). In hippocampal synapses, where GAMMA8 is highly expressed (Rouach et al., 2005; Yamasaki et al., 2016), this pedestal AMPA current was found to be widespread, with a sparse and flat spatial distribution. (Pampaloni et al 2020). Interestingly, different synapses within the same neuron showed different levels of pedestal response, and activation of synapses that displayed pedestal response could reliably trigger action potentials (Pampaloni et al 2020). Thus, TARPs could provide a unique, NMDAR-independent postsynaptic mechanism for altering AMPAR responses in individual synapses, and could have unique roles in neural function. However, the exact electrophysiological properties of many TARP-AMPAR complexes remain largely unstudied.

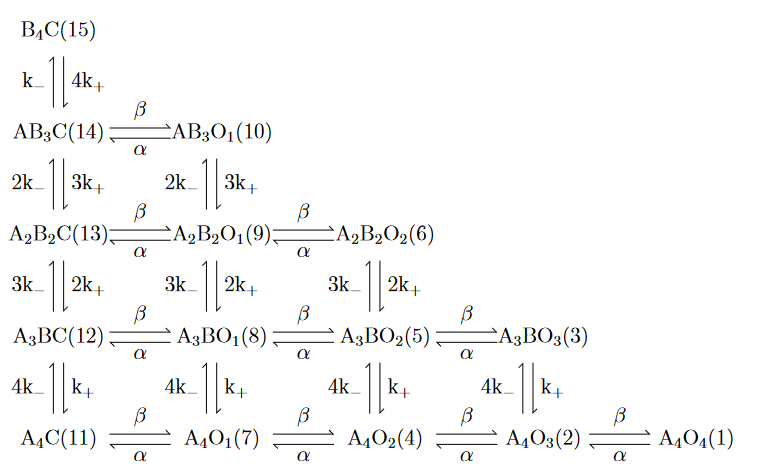
TARPs have also been shown to regulate the polyamine block of AMPARs. In physiological conditions

In this work, I set out to electrophysiologically characterize the combination of homotetrameric GluA4 AMPARs with the TARP GAMMA2. To this effect, I used a construct that consisted of GluA4 linked at the C-terminus via a xxxxxx linker to the xxx-terminus GAMMA2 (A4G2). This ensured equal stoichiometry of both proteins, and complete association GAMMA2 to every GluA4 subunit, reducing excess TARP toxicity. As control, I used homotetrameric GluA4 AMPAs (A4). In addition, to learn patching and to serve as a further control, I used homotetrameric GluA2 AMPARs (A2). I expressed these receptors in HEK293 cells and recorded their macroscopic glutamate responses from outside-out patches.

* 1. **Exact gating mechanisms of AMPAR**

Electrophysiological measurements from single ion channels provide a unique opportunity for studying the exact mechanisms underlying gating and other processes. The amplitude distribution of traces can directly give insight to possible subconductance states. Eleven years before the first full-length crystal structure (Sobolevsky et al 2009), single channel recordings revealed its tetrameric arrangement: Rosenmund et al 1998 demonstrated four distinct subconductance states of (Native?xxx) AMPARs during a stepwise activation protocol in single channel recordings. These four subconductance states have been proposed to correspond with four separate open conformations of the receptor, one for each agonist-bound subunit.

In addition, further states can be resolved by examining the different exponential components of time-dwell distributions for open and shut states in idealized traces. By defining hidden Markov models of receptor activation, predicting the time dwell distributions for these models, and fitting them to the observed distributions, one can even calculate rate constants for transitions between proposed states. (Fig 2)



***Fig 2. Proposed Markov Model for AMPAR sublevel activation.*** *AiBj indicates that i agonist-bound and j unbound subunits comprise the receptor, with ALPHA and BETA being the agonist binding constant. O1-O4 indicates the subconductance state and C the closed state. Adapted from Kuru (xxxx)*

Combining results from structural analysis, single channel recordings and molecular dynamics simulations has allowed for a detailed description of the conformational rearrangements that lead to AMPAR gating and desensitization. The degree of LBD clamshell closure is agonist dependent, has been shown to directly influence open probability, and correlates with gating (Armstrong and Gouaux, 2000). When the clamshell closes, the D1 lobes of the LBD dimerizes, pulling open the

The xxx CTZ, binds to and stabilizes the D1 dimerized

While initially the T1 mutant characterized in …. Was used for xxx.

In NMDARs, two pre-active conformations have been demonstrated, with rate constants of xxx (source). Because WT AMPARs activate much too fast to resolve pre-active states, T1’s slower activation kinetics have facilitated the study of these pre-active states. In his 2021 dissertation, Sebastian Braunbeck recorded from single AMPARs, WT and T1, in the presence of CTZ to block desensitization and either zinc or EDTA (a zinc chelating agent). He was able to resolve three different pre-active states in the first latency distribution of T1 mutant AMPARs in zinc and CTZ.

His recordings provide a rich data set, from which further insights can be gained. In this work, using his data, I set out to find a working definition of the first event, to characterize the distribution of idealized amplitude of the first event, and to find the first latency distributions separately for each of the first event amplitudes.

**2 Materials and Methods**

**2.1 Cell Culture**

The human embryonic kidney 293 (HEK293) cell line was used for all patch clamp experiments. All cells were cultured in Modified Eagle Medium (MEM) supplemented with 6% fetal bovine serum (FBS) in T-25 flasks and incubated at 37ºC and 5% CO2. Cells were split approximately twice a week, when the confluency reached ~90%. The cells were washed with 5 mL of Dulbecco’s phosphate buffered saline (DPBS) and incubated on 250 µL 0.05% Trypsin / 0.02% EDTA at 37ºC for 3 minutes. Afterwards the cells were suspended in 10 mL MEM + 6% FBS to stop trypsination, after which they were centrifuged at 100g for 5 minutes. Supernatant was discarded and the cells were resuspended in 5 mL MEM + 6% FBS and split 1:7 - 1:11 (depending on confluency) into a new T-25 flask.

Two days before transfection, 30 – 200 µL of cell suspension (depending on confluency) were seeded onto a ø25 mm dish containing 2 mL MEM + 6% FBS and ~4 ø10 mm coverslips coated with Poly-L-Lysine. These coverslips had been previously sonicated for 15 minutes in 70% ethanol and had been kept in 99% ethanol at room temperature. For coating, coverslips were flamed and put into ø35 mm dishes. Subsequently, ~200 µL of 0.01% Poly-L-Lysine solution were added to the coverslip, incubated at room temperature for 3 minutes, and removed. After washing twice in 2 mL DPBS, the dishes with coated coverslips were stored at 4ºC for up to 4 weeks.

**2.2 Transfection**

6 µL of xxx polyethylenimine (PEI) solution were added to 192 µL of OPTI-MEM and incubated for 3 minutes at room temperature. 2 µL of 1 ng/µl plasmid solution totalling 2 ng DNA were added and incubated for another 20 minutes at room temperature. The total mixture was then added dropwise to the cells and incubated for 5-12 hours at 37ºC. Afterwards, the transfection medium was removed, the cells were washed twice in 2 mL DPBS prior to adding 2 mL of fresh medium supplemented with 30 µL 2 mM NBQX disodium solution in water for a total concentration of 30 µM.

The plasmids that were used for the GluA2 condition contained GluA2 (Q) flip and GFP, both under an IRES promoter. The GluA4 condition contained GluA4-flip under an IRES promoter. The GluA4+GAMMA2 condition contained GluA4-flip linked at its C-terminus via a 12 amino acid linker (Ser-Arg-Gly-Gly-Gly-Gly-Gly-Gly-Thr-Gly-Ala-Thr) to the N-terminus of GAMMA2 without the start codon and GFP, both under an IERS promoter.

**2.3 Electrophysiological setup**

Recordings were done 16-48 hours after transfection. Data was collected on the same electrophysiology setup using an Axopatch 200B patch clamp amplifier and amplifier output was monitored on a Tektronix TBS1102B digital oscilloscope. Digitization was performed using a HEKA InstruTECH ITC-18 interface.

Patch pipettes were pulled using a Sutter P-1000 pipette puller from World Precision Instruments TW150F3 3-inch thin-wall borosilicate glass capillaries, with an outer diameter of 1.5 mm and an inner diameter of 1.12 mm. Electrodes had previously been made from Teflon coated silver wire tipped with a silver chloride pellet. The head stage containing the electrode holder was moved using a Scientifica PatchStar micromanipulator. Pipettes typically had resistances of 3-6 MΩ, and were mounted onto a G23 Instruments electrode holder after being filled with intracellular solution (Table XXX). To block the inward xxxx rectification of GluA4 in the presence of polyamines xxxxxxxxx

Solution exchange was achieved using a Physik Instrumente piezoelectric stack actuator, which received XXX V pulses from the acquisition software, transmitted by a Siskiyou controller. The actuator moved a perfusion tool, which had been made in the lab as laid out in detail in Plested and Poulsen 2021 (SOURCE), exposing the stationary patch pipette to two different extracellular solutions (Table XX). Solution 2 contained xxxx glutamate for receptor activation and xxx sucrose to make it easier to distinguish under the microscope.

For patching, coverslips containing cells were removed from the incubator and placed into a fresh ø35mm dish containing Solution 1 mounted on a Zeiss Axiovert 100 microscope in a TMC Faraday cage on a TMC vibration-free table. eGFP fluorescence was examined using blue excitation light emitted by an X-Cite series 120Q. Healthy looking, transfected cells were selected by inspection. After an outside-out patch had been excised, the solution flow through the perfusion tool was started. After the recordings, the bath solutions were replaced to keep extracellular glutamate solutions low. All data was acquired using the AxoGraph acquisition software (AxoGraph Scientific, John Clements) at 40kHz after passing through an 8-pole Bessel filter at 10 kHz. The speed of solution exchange and positioning of the patch pipette was tested by applying the solutions to an open tip patch after recording.

**2.4 Electrophysiological recordings**

For recording, several different protocols were used. To study rise time, peak current, and steady state current, as well as to investigate the kinetics of both desensitization and decay of the receptor response, a 500 ms pulse of glutamate was applied 20 times. To measure the decay of a more physiologically relevant activation, a 2 ms pulse of glutamate was applied 20 times. To measure the recovery from desensitization, a protocol with an initial 500 ms pulse of glutamate was applied, followed by eleven 25 ms pulses, with incrementally more time in between each pulse, from 25 ms to xxx ms, in steps of xxx ms. This incremental protocol was applied 10 times. To measure desensitization in a more physiologically relevant manner than sustained glutamate exposure, 2 ms glutamate pulses were applied at 10 Hz, 20 Hz and 50 Hz for 5 seconds, 5 times respectively. All protocols were recorded at both a holding potential of -60 mV, as well as +50 mV, in so far as this was possible for each patch.

To measure the voltage dependency of the current, the holding potential of the patch was successively set to a series of values: (-100 mV, -80 mV, -60 mV, -40 mV, -20 mV, 0 mV, 20 mV, 40 mV, 60 mV, 80 mV and 100 mV), xxx ms after which a xxx ms glutamate pulse was applied.

Sample recordings can be found in the appendix. xxxxxx

**2.5 Data Analysis**

Macroscopic current recordings were selected, averaged and baseline corrected in AxoGraph. Subsequently, statistical calculations like rise time, peak current, steady state, as well as event detection were all performed using IgorPro64 (xxxxx) and compiled in Microsoft Excel (xxxxx). Similarly, xxxxx exponentials were fitted to the trace in IgorPro64, ALPHA and TAU values were exported into Microsoft Excel, where the weighted TAU was calculated using the following formula: xxxx The code for further analysis and data visualization was written in python (version xx, xxx), using the python matplotlib library (xxxx), and is publicly available on the GitHub repository (https://github.com/LukBorn/ASCAM)

**2.6 Single channel analysis**

All analysis for the single channel project was written in python (source). For the majority of the final data analysis, the python program ASCAM (source) was used. Data and results were visualized using the matplotlib library (xxxxx).

For this project, all data, with a convenient overview spreadsheet, was available on the lab server, and had been previously recorded and analyzed as laid out in (Braunbeck 2021). In brief, traces were selected, gauss filtered at 1000-2000 kHz and baseline corrected in ASCAM before further analysis. Activation time was determined in ASCAM via automated threshold crossing algorithm, with a different threshold determined for each recording (~-0.5 pA). Idealization was also performed via automated threshold crossing algorithm, with one closed and four open states, with conductances in the range 0, -0.6, -1.2, -1.8, -2.5, determined individually for each recording, with a resolution of 130 µs, meaning that events shorter than 130 µs were not counted as events.

In total, five total usable recordings existed of the T1 in EDTA+CTZ condition. Analysis of activation time had been done for all five recordings, summarized in a .csv file containing activation time for each episode. Idealization had been conducted on three of five recordings, and a list of all events containing amplitude, start and end time, duration and episode number. Similarly, for the T1 in zinc+CTZ condition, nine usable recordings existed, of which seven had been analyzed for activation time and three had been idealized. For first event analysis, only those recordings that had both activation time and events .csv were utilized, totaling three per condition. Of both the WT in EDTA+CTZ condition, and the WT in zinc+CTZ condition, several recordings existed, none of which had been idealized however, so they were not included in further analysis.

**Results**

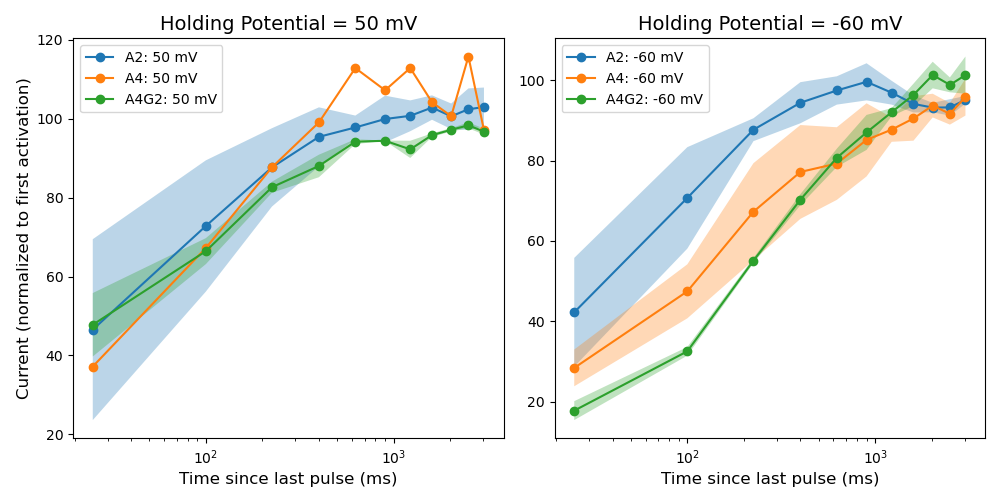
**3.1 GluA4 gamma2 tandem kinetics**

No significant differences existed between the rise times of all conditions (Fig X. left), determined by Kruskal-Wallis test (p-value = 0.26128). All rise times fell in the range of 0 – 2.5 ms which was close to the literature value of 200 - 650 µs (xxxx), except for one outlier in the A4 -60mV condition. For peak current (Fig X. middle), no significant differences could be found between any of the conditions, determined by Kruskal-Wallis test (p-value = 0.07955). As the peak current is mostly a direct result of patch quality, this result can be taken as a confirmation of the comparability of the data gathered. The steady state current (Fig X. right) also shows no significant difference between all conditions, determined by Kruskal-Wallis test (p-value = 0.26088).

A graph of different sizes and colors

Description automatically generated with medium confidence***Fig X. left: Rise time.*** *Time (ms) from 10% to 90% of peak current for the activation upon glutamate exposure.* ***middle: Peak current.*** *Peak amplitude (pA) of the activation upon glutamate exposure.* ***right: Steady state current.*** *Mean current upon reaching equilibrium after receptor desensitization, normalized to peak current, in %. A2 50mV: n=3. A2 -60mV: n=4. A4 50mV: n=2. A4 -60mV: n=12. A4G2 50mV: n=4. A4G2 -60mV: n=5.*

The incremental protocol, with progressively more time in between glutamate pulses, allows for an easy visualization for the recovery of desensitization, starting with completely desensitized receptors after a long initial glutamate pulse. When the exposure to glutamate is ended, some receptors recover from desensitization and can open at the next glutamate pulse. The longer in between each pulse, the more receptors can recover, and the higher the current at the next event is. The speed of this recovery corresponds to the average lifetime of the desensitized state after the end of glutamate exposure. At 50 mV, there are no obvious differences between the recovery curves, mostly due to the A4 condition having n=1, and the A2 condition having large variance (Fig X. left). However, at -60 mV, the A2 condition shows faster than the A4 condition, and both are faster than the A2G4 condition (Fig X. right).

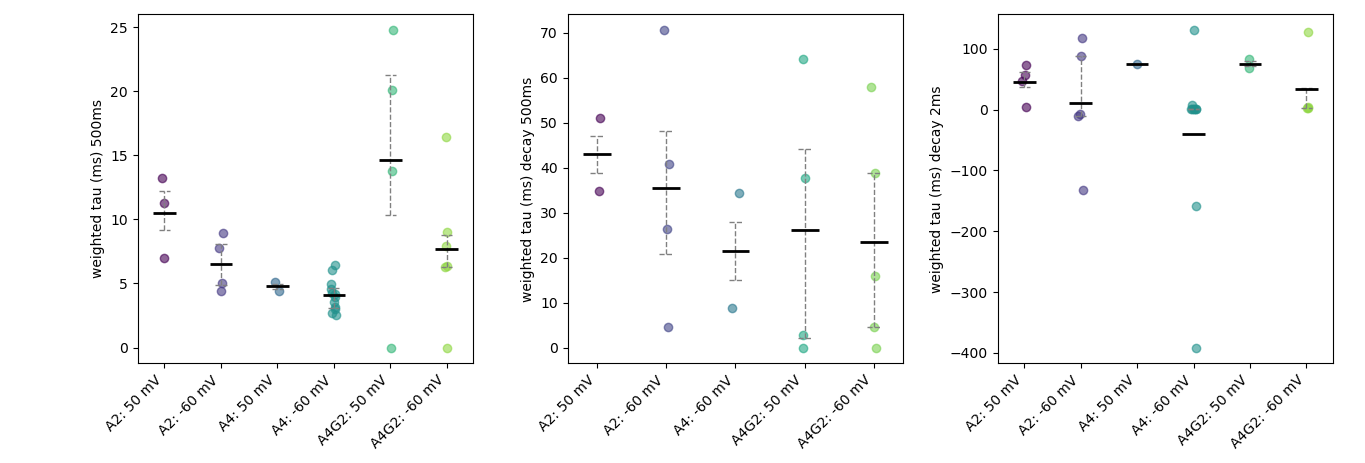
****

***Fig X. Recovery from desensitization.*** *Incremental protocol: Peak current of each 25 ms glutamate pulse, excluding initial xx ms pulse. Normalized to peak current of initial 500 glutamate pulse, in % plotted against log time since last pulse in ms. A2 50mV: n=3. A2 -60mV: n=3. A4 50mV: n=1. A4 -60mV: n=5. A4G2 50mV: n=3. A4G2 -60mV: n=3.*

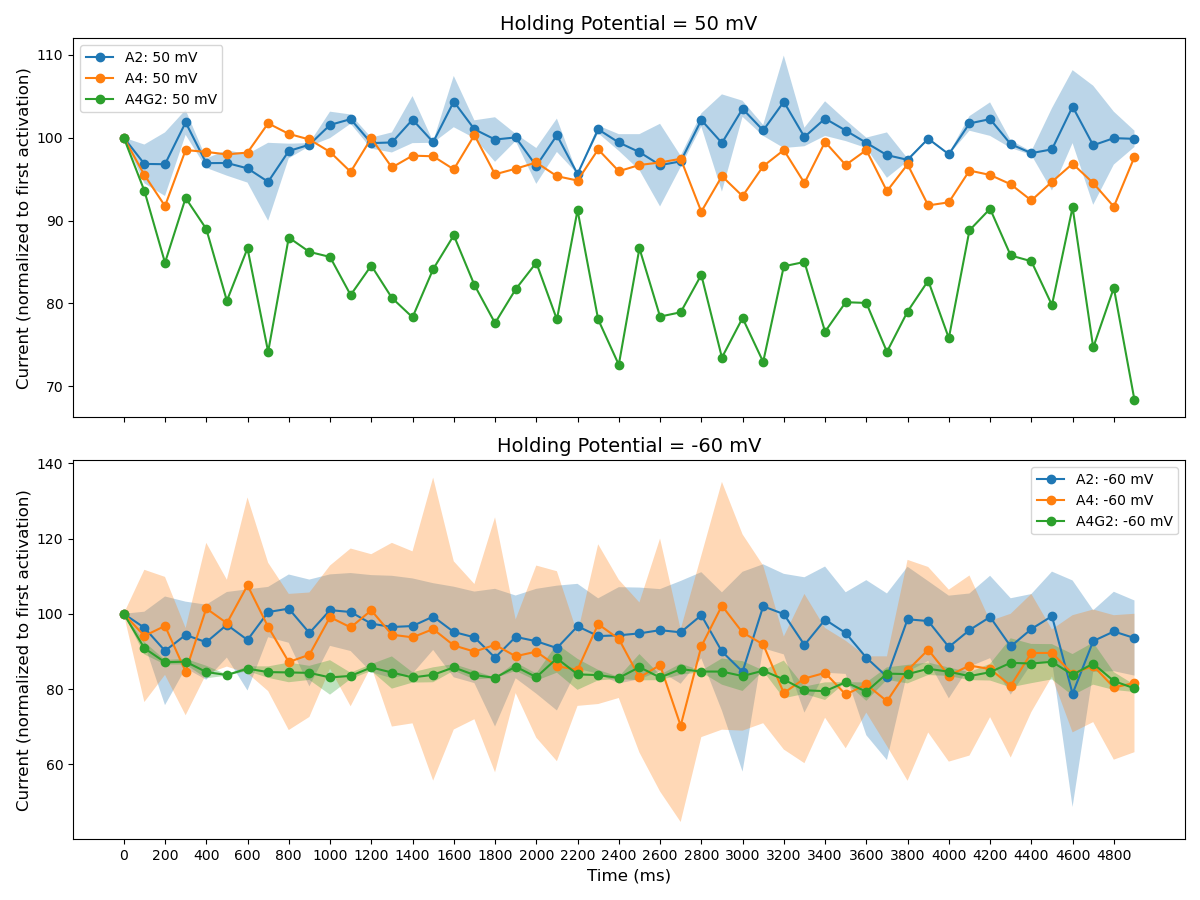
The decay of the trace during glutamate exposure is driven by desensitization of the receptor. Significant differences between the weighted TAU of the exponentials fitted to these traces were found between the A4G2 condition at -60 mV and the A4 condition -60 mV, determined by Mann-Whitney U test (p-value = 0.04148), the A2 condition at 50 mV and A4 condition at -60 mV, determined by Mann-Whitney U test (p-value = 0.00440), and the A2 condition at -60 mV and the A4 condition at -60 mV, determined by Mann-Whitney U test p-value = 0.02967. Notably, all comparisons include the A4 condition at -60 mV for which 12 separate recordings were analyzed. With such a high n compared to all other samples, finding significant differences here is unsurprising.

The decay of the trace after the end of glutamate exposure corresponds to the lifetime of the open state after the end of glutamate exposure. This curve should be dominated by the unbinding constant of glutamate from the receptor, as open states without glutamate bound do not exist in any meaningful capacity. No significant differences were found in the weighted TAU of these decay curves, determined by Kruskal-Wallis test (p-value = 0.80976).

The decay after a 2 ms glutamate pulse more closely resembles an actual physiological activation of the receptors, as the glutamate concentration in the synaptic cleft rapidly decreases after synaptic release events. The component processes in this decay curve are therefore both desensitization, and unbinding of glutamate. No significant differences were found in the weighted TAU of these curves, determined by Kruskal-Wallis test (p-value = 0.11585). Negative values for the weighted TAU were caused by incorrect baseline correction for the traces of these recordings, leading to exponentials being fit to negative values.

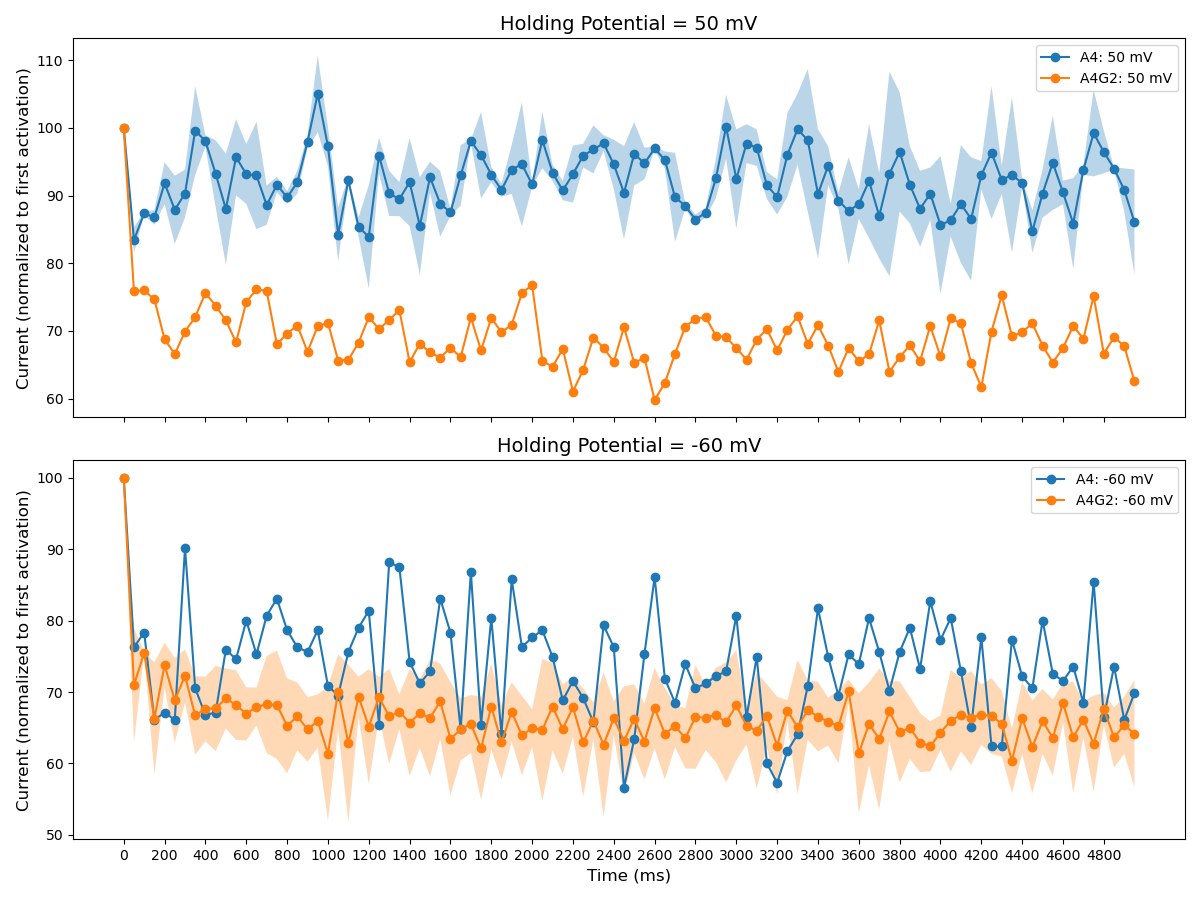


***Fig X. Kinetics. A: Desensitization.*** *Weighted TAU (ms) of exponentials fitted to trace during 500 ms glutamate pulse. A2 50mV: n=3. A2 -60mV: n=4. A4 50mV: n=2. A4 -60mV: n=12. A4G2 50mV: n=4. A4G2 -60mV: n=5.* ***B: Decay.*** *Weighted tau (ms) of exponentials fitted to trace after 500 ms glutamate pulse. A2 50mV: n=2. A2 -60mV: n=4. A4 -60mV: n=2. A4G2 50mV: n=2. A4G2 -60mV: n=4.* ***C: Desensitization.*** *Weighted tau (ms) of exponentials fitted to trace after 2 ms glutamate pulse. A2 50mV: n=4. A2 -60mV: n=5. A4 50mV: n=1. A4 -60mV: n=10. A4G2 50mV: n=3. A4G2 -60mV: n=3.*

****During repeated stimulation, receptors become activated and desensitized at each stimulation. Depending on the decay rate of the desensitization, a certain percentage of receptors remain desensitized and will not be activated at the next stimulation. Longer desensitization leads to less receptors available at next stimulation, so a larger drop in conductance at the beginning of the recording, and a lower normalized current once the system has reached an equilibrium. During 10 Hz stimulation, A4G2 condition recordings at both holding potentials show weak but observable desensitization, apparent as a reduction in normalized peak current for each stimulation at the start of the recording, and a leveling out of these normalized peak currents over time (Fig X). Compared to both controls at 50 mV holding potentials, this is a stonger desensitization (Fig X. top). The variance between recordings for each of the control recordings at -60 mV is too large for an assessment of the shape of the curve and comparison with A2G4 (Fig X. bottom).

For 20Hz stimulation, no recordings of the A2 conditions were of high enough quality to analyze fully. The recordings show desensitization across all conditions (Fig X.) with more desensitization when compared to the 10 Hz protocol (Fig X.). This is to be expected, as the less time between each pulse means more receptors are desensitized state at the next pulse, so lower currents. Across both holding potentials, the A4 condition shows weaker desensitization than the A4G2 condition (Fig X). For 50Hz stimulation, no recordings in any condition were of high enough quality to be fully analyzed.

***Fig X. Desensitization during 10Hz stimulation.*** *Peak current of each 2 ms glutamate pulse, normalized to peak current of initial glutamate pulse, in %. A2 50mV: n=2. A4 50mV: n=1. A4G2 50mV: n=1. A2 -60mV: n=3. A4 -60mV: n=2. A4G2 -60mV: n=2.*

** *Fig X. Desensitization during 20Hz stimulation.*** *Peak current of each 2 ms glutamate pulse, normalized to peak current of initial glutamate pulse, in %. A4 50mV: n=2. A4G2 50mV: n=1 A4 -60mV: n=1. A4G2 -60mV: n=2.*

The voltage dependency of the receptor is determined by plotting the peak current upon stimulation by glutamate pulse for a range of holding potentials, resulting in an IV curve. The curve of the A2 condition is fairly linear across the whole range of holding potentials (Fig. X left). Recordings for this condition had no spermine in the intracellular solution, so no polyamine block at low absolute holding potentials. The A2 condition was not a good control, since the A4 condition or the A4G2 condition did have spermine in the intracellular solution. It mostly served as an introduction to patching. The A4 condition shows a very nonlinear IV curve, with low peak currents close to the xxxxxx. This is characteristic shape of the polyamine block of ionotropic glutamate receptors. (Bowie xxxxxx) The A4G2 condition, however, again shows a linear voltage dependency, indicating that the GAMMA2 subunit modulates the polyamine block.

**A graph with lines and numbers

Description automatically generated**

***Fig X. Voltage Dependency: I-V curves****. Peak current of glutamate pulse at each holding potential, normalized to peak current of glutamate pulse at -80 mV. A2: n=3. A4: n=11. A4G2: n=3.*

**3.2 Single channel analysis**

Initially, for each episode, an idealized trace was reconstructed from the available events list .csv file. The activation time was also taken from the available .csv file, and to take into account the idealization, half the resolution time (65 µs) were added. When plotting all episodes from one recording of T1 in EDTA+CTZ in a scatter plot with marginal histograms (Fig X left), it becomes apparent that, for most episodes, the amplitude of the idealized trace at the predefined activation time is 0. As the first event must, by definition, not have an amplitude of 0, this definition of a first event was unsatisfactory.

In a second attempt to define the first event, for each episode, again using the available predefined events list and activation table, the first event after first activation plus dead time that had a drop in absolute amplitude was set as first event. These events were deemed “maximal first events” (Fig X. right) While this definition solved the problem of firsts event with a conductance of 0.0, as there was no way an event with a conductance of 0.0 could be followed by a drop in absolute conductance, it was also unsatisfactory. The time difference between the first activation and the start of the event defined as first event could be quite large, putting into question the purpose of the first activation detection itself. The event defined as first event could be up to four events after the event containing the first activation time, if the event containing the first activation time had an amplitude of 0.0 as well as no drop in absolute amplitude until the highest subconductance state. Additionally, there was no mechanistic significance to the first drop in subconductance, no reason this event should hold any more information than the events the idealization algorithm detected before the maximal first events.

**A comparison of a graph

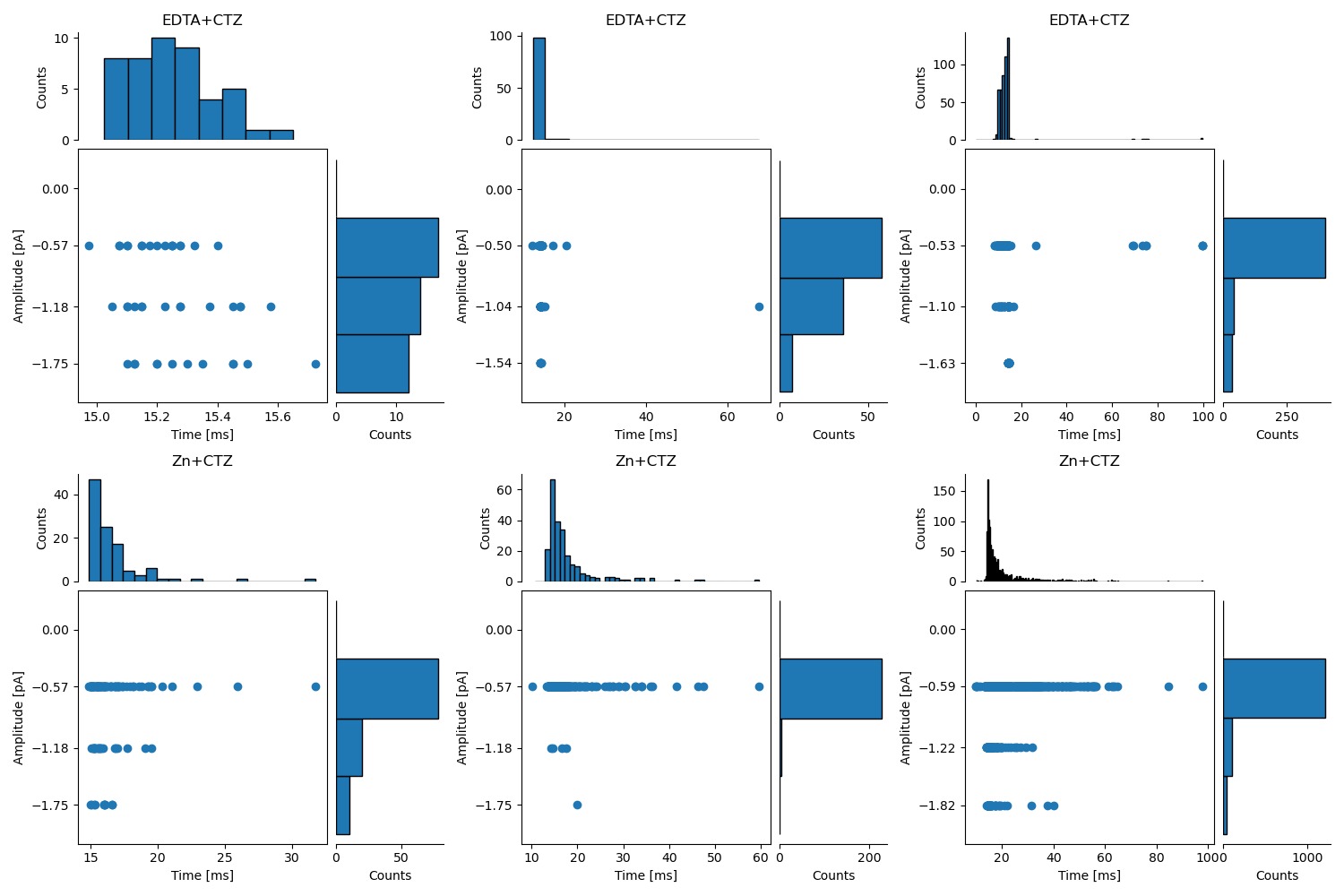
Description automatically generated with medium confidence*Fig X. left: First event amplitude.*** *Absolute amplitude in pA of predefined idealized trace at predefined activation time in ms.* ***right: Maximal first event amplitude.*** *Absolute amplitude in pA and start time in ms of nonzero event before first drop in absolute amplitude in idealized trace after t0. Data from recording 19 on 25.10.2017 (T1 mutant in EDTA and CTZ)*

To be able to better develop the first events analysis, as well as to have this analysis available to others with similar research questions, further implementation was done directly in ASCAM. First, to solve a different problem that was encountered with many episodes, the parameter “exclusion time” was added to the first activation analysis page in ASCAM. For many episodes, the trace crossed the first activation threshold at the very start of the episode, presumably as an artefact of the filtering. This resulted in many episodes having a falsely detected first activation, and the episode needing to be excluded or manually reviewed. By excluding the first couple milliseconds from the threshold crossing algorithm, a lot more episodes could be used than previously, without manual review.

To detect the first event, first the first activation was determined via threshold crossing as before. Then, dead time to account for the resolution of the idealization was added to determine the first event time point *t*. If the amplitude of the idealized trace at *t* was not 0, the event containing *t* was defined as first event. Otherwise, if the idealized trace at *t* was 0, the next event was defined as first event. A table of first event times, amplitudes and episode number could then be generated and exported. For the following data, only the previously idealized recordings were used. Threshold for first event detection was set at -0.5 pA and exclusion time at 10 ms. The subconductance state amplitudes were kept the same as for the previous idealizations. First events were detected for every recording that had previously idealized (Fig X).

Across all recordings, the most common first event amplitude is the lowest subconductance state, especially in the recordings with the most episodes (Fig X top right, bottom right). In the Markov model of receptor activation, this is the is the open state with most conformations (7-10), as well as the one closest to the closed state, so that it is visited first should be expected. Notably, for the T1 in EDTA+CTZ condition, the amplitude distributions are flatter, with more episodes with first events of a higher subconductance state. This condition is expected to have faster activation kinetics than the zinc+CTZ condition, so intermediate states with a dwell time too short to pick up un the idealization are more common, leading to bigger jumps in the idealized trace. This results in more first events with a high amplitude, leading to a flatter distribution of first event amplitudes.

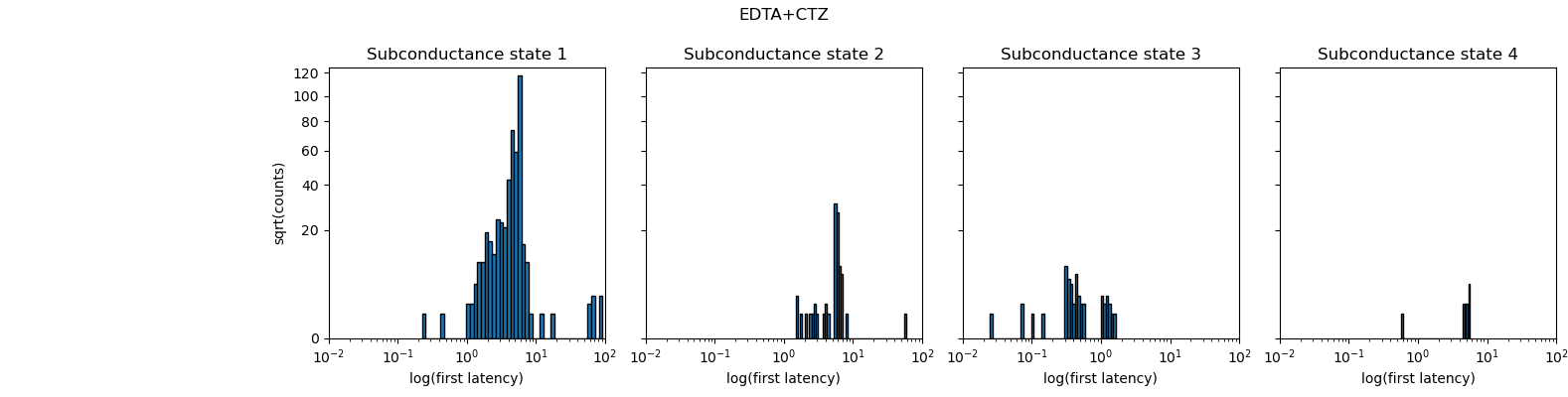
Transitioning from closed to open state is a process that can be assumed to have same probability across all time points, resulting in an exponential distribution of first latencies (Fig X). In addition, first events opening to a higher subconductance states tend to be much earlier (Fig X). If the openings to different subconductance states have similar exponential distributions, one would expect to find a lot more early openings with a smaller sample size, which higher amplitude first events tend to have. Indeed, in the recording with the most similar number of openings for each subconductance state (Fig X top left), the distribution of the activation times between are more similar between first event amplitude than in other recordings.

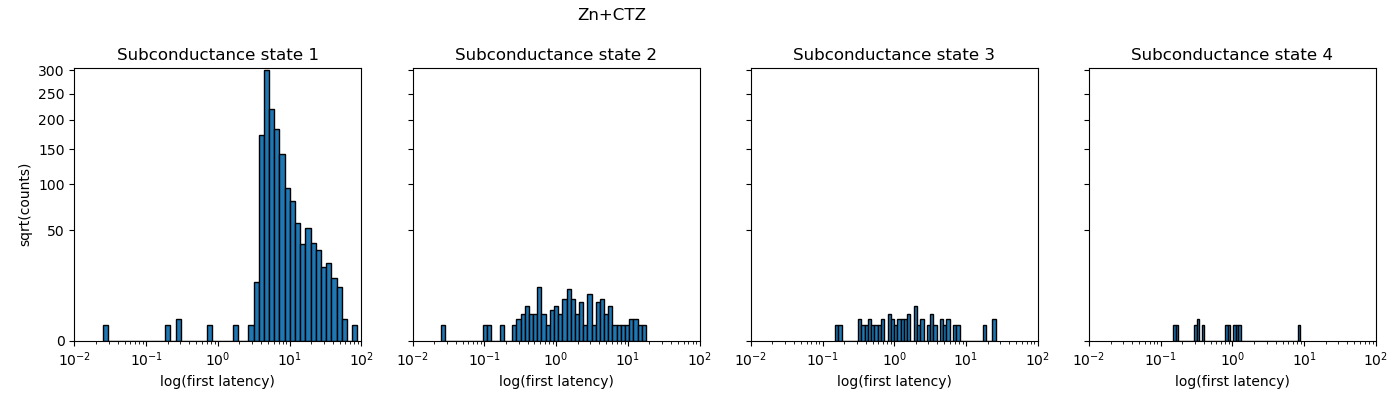
****

***Fig X. First event amplitude.*** *Amplitude in pA and start time in ms of first event. For T1 in EDTA and CTZ: Recording 19 from 25.10.2017 (left), recording 24 from 25.01.2018 (middle) and recording 4 from 18.05.2018 (right). For T1 in zinc and CTZ: Recording 24 from 24.01.2018 (left), recording 0 from 26.04.2018 (middle) and recording 14 07.05.2018 (right).*

**3.2.4 First latency histograms for each initial subconductance state**

When examining the first latency distributions separately for each subconductance state and plotting them on a log square root scaled histogram, what becomes most readily apparent is the much slower activation times of the zinc+CTZ condition. All the distributions are further right on the logarithmic time scale in the zinc+CTZ condition compared to the EDTA+CTZ condition (Fig. X, Fig. X). In the histograms for subconductance state 1, there seem to be some humps in the distributions for both conditions, indicating there might be a multimodal distribution with more than one component. However, without fitting exponentials to these distributions, no quantitative assessment of the number or time constants of the components in these distributions can be drawn. For higher subconductance state first events, there is not enough data for any good conclusions to be drawn from the distribution, for either condition (Fig. X middle and right, Fig. X middle and right).



***Fig X. T1 mutant in EDTA and CTZ.*** *First latency histogram for each initial subconductance state, with log(ms), square root(counts) scale. State 1: ~-0.55 pA. State 2: ~-1.10 pA. State 3: ~-1.61 pA. State 4: ~-2.21 pA. Data pooled from 3 recordings.*

***Fig X. T1 mutant in zinc and CTZ.*** *First latency histogram for each initial subconductance state, with log(ms), square root(counts) scale. State 1: ~-58 pA. State 2: ~-1.20 pA. State 3: ~-1.77 pA. State 4: ~-2.37 pA. Data pooled from 3 recordings.*

**4 Discussion**

**4.1 Characterization of the GluA4 GAMMA2 tandem**

No difference in any kinetics.or riste time etc

Slower recovery from desensitization, more desnsitization

Linear IV in GluA4 GAMMA2 vs whatever rectiying

I didnt find any lower steady state, no significant differences

Bad control, not enough recordings. Maybe it’s the tandem, or the expression, or my patching skills

Previously, when co-expressing GluA2 or GluA4 with GAMMA2 in Hek293 cells and recording using incremental, 10Hz and 20Hz protocols, a distinct type of electrophysiological response deemed superactivation was observed. Superactivation was characterized by an increase in peak current and current during repeated and continued state stimulation, respectively, after desensitization.

In addition, the presence of TARPs causes weaker desensitization.

This was proposed a distinct superactivated state, which can only be achieved in the presence of TARPS and has far slower activation kinetics than the normal open state (Carbone, Plested 2016).

These findings do not match with my observations. The A4G2 condition showed stronger desensitization, and slower recovery from desensitization compared to controls, in

This difference could be caused

In other work there is very much pedestal opening, especially in single channel.

Just patch it all again or try a different construct.

**4.2 single channel analysis**

The two definitions that were unsatisfactory

**Limitations**

Effect of the resolution on the idealization, in general the idealization is quite flawed probably

Noise of the setup, limited samples etc

**Future directions**

Look at the distribution of first latencies for each first open state -> how many (resolvable) shut states before going into the corresponding open state, fitting exponentials, looking at wild type.

Expanding analysis of the trace to dwell times of more complex events than dwell times

Todos in ascam

**References**