GluK1 kainate receptors regulate synaptic population activity and plasticity in the amygdala

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HELSINGIN YLIOPISTO
HELSINGFORS UNIVERSITET
UNIVERSITY OF HELSINKI

Tiedekunta – Fakultet – Faculty	Koulutusohjelma – Utbildingsprogram – Degree Programme
Bio- ja ympäräristötieteellien tiedekunta	Neurotieteen maisteriohjelma

Tekijä – Författare – Author

Joni Haikonen

Työn nimi – Arbetets titel – Title

GluK1 kainate receptors regulate synaptic population activity and plasticity in the amygdala

Oppiaine/Opintosuunta – Läroämne/Studieinriktning – Subject/Study track

Neurotiede

Työn laji – Arbetets art – Level	Aika – Datum – Month and year	Sivumäärä – Sidoantal – Number of pages
Maisterintutkielma	6.5.2019	36

Tiivistelmä – Referat – Abstract

Englanniksi (in English):

Kainate receptors are known to regulate neuronal function in the brain (Li, H., & Rogawski, M. A. (1998), Braga, M. F. et al. (2004), Lerma & Marques (2013), Carta, M (2014)). In the amygdala, they have been shown to affect synaptic transmission and plasticity, as well as glutamate and γ-aminobutyric acid (GABA) release (Li, H. et al. (2001). Braga, M. F. et al. (2003), Braga, M. F. et al. (2009), Aroniadou-Anderjaska, V. et al. (2012), Negrete-Díaz, J. V. et al. (2012)), however, their role during development of the amygdala circuitry is not known. In the present study, we wished to understand how GluK1 kainate receptors regulate synaptic population activity and plasticity in the developing amygdala by using extracellular field recordings in P15-18 Wistar Han rat pup brain slices. Since field excitatory postsynaptic potentials (fEPSPs) are not commonly measured from the amygdala, we first sought to pharmacologically characterize the basic properties of the extracellular signal, recorded from the basolateral amygdala in response to stimulation of the external capsulae (EC). Having confirmed the validity of the fEPSP as a measure of postsynaptic population response, we were able to show that blocking GluK1 with (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (ACET), a selective GluK1 antagonist, had no effect on the fEPSP. Furthermore, activation of GluK1 with RS-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid (ATPA), a GluK1 agonist, reduced the amplitude of the fEPSP, without affecting its slope, suggesting an increase in inhibitory signaling within the network. Blocking GABAergic activity with GABAA- receptor antagonist picrotoxin significantly reduced the effects of ATPA. Additionally, the increase in inhibitory signaling due to the activation of GluK1 was confirmed with whole-cell voltage clamp, by measuring spontaneous inhibitory postsynaptic current (sIPSC) frequency. Activation of GluK1 heavily increased sIPSC frequency in the basolateral amygdala neurons. Finally, we were also able to show that activation of GluK1 with ATPA strongly attenuates LTP induction. These results show that GluK1 kainate receptors play a vital role in the modulation of synaptic transmission and plasticity in the developing amygdala.

Suomeksi:

Kainaattireseptorit säätelevät hermosolujen toimintaa aivoissa (Li, H., & Rogawski, M. A. (1998), Braga, M. F. et al. (2004), Lerma & Marques (2013), Carta, M (2014)). Mantelitumakkeessa näiden reseptorien on osoitettu vaikuttavan synaptiseen signalointiin ja plastisuuteen, sekä glutamaatin ja γ-aminobutyyrihapon (GABA) vapautumiseen synaptisesta päätteestä (Li, H. et al. (2001). Braga, M. F. et al. (2003), Braga, M. F. et al. (2009), Aroniadou-Anderjaska, V. et al. (2012), Negrete-Díaz, J. V. et al. (2012)), mutta niiden vaikutusta mantelitumakkeen hermoverkkojen kehitykseen ei tunneta. Tässä tutkielamssa halusimme ymmärtää kuinka GluK1 kainaatti reseptorit säätelevät synaptista populaatio aktiivisuutta ja plastisuutta kehittyvässä mantelitumakkeessa, mittaamaalla solunulkoisia kenttäpotentiaaleja P15-18 ikäisten Wistar Han rottien aivoleikkeistä. Koska solunulkoisia kenttäpotentiaaleja ei usein mitata mantelitumakkeesta, määritimme mittaamamme kenttäpotentiaalin ominaisuudet farmakologisesti. Kun kenttäpotentiaalin validiteetti oli määritetty, kykenimme osoittamaan, että GluK1 kainaattireseptorien toiminnan esto S)-1-(2-Amino-2-karboxyethyyli)-3-(2-karboxy-5-fenyylithiofeny-3-yli-metyyli)-5-metyylipyrimidiini-2,4-dioni (ACET) ei aiheuttanut merkittäviä muutoksia kenttäpotentiaalissa. GluK1 aktivaatio GluK1 agonistilla RS-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) proprionihappolla (ATPA) madalsi kenttäpotentiaalin amplitudia, vaikuttamatta sen konduktiojyrkkyyteen, viitaten inhibitorisen signaloinnin lisääntymiseen hermoverkossa. Estämällä GABAergiset reseptorit pikrotoksiinilla (GABAA-reseptori antagonisti) ATPAn vaikutukset kenttäpotentiaalin amplitudiin vähenivät merkittävästi. Lisäksi, varmistimme että GluK1 aktivointi ATPAlla lisää inhibitorista signalointia mantelitumakkeessa, mitaamalla spontaanin inhibitorisen signaloinnin frekvenssiä kokosolun virtalukituksella. GluK1 aktivaatio kasvatti spontaanin inhibitorisen signaloinnin frekvenssiä merkittävästi mantelitumakkeen neuroneissa. Lopuksi kykenimme myös osoittamaan, että GluK1 kainaattireseptorien aktivointi ATPAlla estää pitkäkestoisen potentiaation (LTPn) muodostumista. Tuloksemme osoittavat, että GluK1 reseptorit olennaisesti säätelevät synaptista signalointia ja plastisuuttaa kehittyvässä mantelitumakkeessa.

Avainsanat – Nyckelord – Keywords Amygdala, Plasticity, GluK1, Kainate receptors, Wistar-han, Development
Ohjaaja tai ohjaajat – Handledare – Supervisor or supervisors Sari Lauri
Säilytyspaikka – Förvaringställe – Where deposited E-thesis järjestelmä, Helsingin Yliopisto
Muita tietoja – Övriga uppgifter – Additional information

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1. Abstract

Kainate receptors are known to regulate neuronal function in the brain (Li, H., & Rogawski, M. A. (1998), Braga, M. F. et al. (2004), Lerma & Marques (2013), Carta, M (2014)). In the amygdala, they have been shown to affect synaptic transmission and plasticity, as well as glutamate and γ-aminobutyric acid (GABA) release (Li, H. et al. (2001). Braga, M. F. et al. (2003), Braga, M. F. et al. (2009), Aroniadou-Anderjaska, V. et al. (2012), Negrete-Díaz, J. V. et al. (2012)), however, their role during development of the amygdala circuitry is not known. In the present study, we wished to understand how GluK1 kainate receptors regulate synaptic population activity and plasticity in the developing amygdala by using extracellular field recordings in P15-18 Wistar Han rat pup brain slices. Since field excitatory postsynaptic potentials (fEPSPs) are not commonly measured from the amygdala, we first sought to pharmacologically characterize the basic properties of the extracellular signal, recorded from the basolateral amygdala in response to stimulation of the external capsulae (EC). Having confirmed the validity of the fEPSP as a measure of postsynaptic population response, we were able to show that blocking GluK1 with (S)-1-(2-Amino-2-carboxyethyl)-3-(2-carboxy-5-phenylthiophene-3-yl-methyl)-5-methylpyrimidine-2,4-dione (ACET), a selective GluK1 antagonist, had no effect on the fEPSP. Furthermore, activation of GluK1 with RS-2-amino-3-(3-hydroxy-5-tert-butylisoxazol-4-yl) propanoic acid (ATPA), a GluK1 agonist, reduced the amplitude of the fEPSP, without affecting its slope, suggesting an increase in inhibitory signaling within the network. Blocking GABAergic activity with $GABA_A$- receptor antagonist picrotoxin significantly reduced the effects of ATPA. Additionally, the increase in inhibitory signaling due to the activation of GluK1 was confirmed with whole-cell voltage clamp, by measuring spontaneous inhibitory postsynaptic current (sIPSC) frequency. Activation of GluK1 heavily increased sIPSC frequency in the basolateral amygdala neurons. Finally, we were also able to show that activation of GluK1 with ATPA strongly attenuates LTP induction. These results show that GluK1 kainate receptors play a vital role in the modulation of synaptic transmission and plasticity in the developing amygdala.

2. Introduction

2.1 The Amygdala – Form and Function

The amygdala is a teardrop-shaped structure residing within the lower parts of the temporal lobe. It is commonly divided in to the basolateral, cortical and centromedial nuclei and receives input mainly from the afferent cortical and thalamic projections (sensory and memory related information), as well as from hypothalamic and brainstem projections (behavioral information) (Sah et al. (2003)). We will mainly focus on the basolateral nuclei, which can be further divided in to the lateral (LA) and basolateral (BLA) parts. They receive glutamatergic inputs from the cortex via the external capsule (EC) and from the thalamus via the internal capsule (IC) (Mahanty, N. K., & Sah, P. (1999)). These glutamatergic projections innervate a morphologically and functionally diverse array of neurons. The most abundant type of neuron within the lateral and basolateral divisions of the amygdala are pyramidal neurons (~70%), followed by smaller and rounder, non-pyramidal stellate cells (Millhouse, O. E., & DeOlmos, J. (1983)), which were later identified as various types of GABAergic interneurons (McDonald, A. J. (1985), McDonald, A. J., & Pearson, J. C. (1989)).

The amygdala plays an important role in emotional learning processes (LeDoux, J. (2003), McGaugh, J. L. (2004). It has been implicated in regulating fear conditioning and extinction memory, as well as anxiety and stress induced behaviors (Davis, M. (1992), Dityatev, A. E., & Bolshakov, V. Y. (2005), Roozendaal, B. et al. (2009)), through synaptic plasticity within the network (Fanselow, M. S., & LeDoux, J. E. (1999), Blair, H. T. et al. (2001), Dityatev, A. E., & Bolshakov, V. Y. (2005), Pape, H. C., & Pare, D. (2010)). Extensive research has gone in to uncovering the mechanisms that underlie fear-learning in the amygdala. Initial studies simply noted that lesions restricted to the amygdalar nuclei caused severe impairments in fear learning (LeDoux et al. (1990), LaBar, K. S., & LeDoux, J. E. (1996), Majidishad, P. et al. (1996), Goosens, K. A., & Maren, S. (2001)). In conjunction with these results, N-methyl-D-aspartate (NMDA) receptors were observed to be necessary for fear conditioning to take place (Maren, S., et al. (1996), Lee, Hongjoo J., et al. (2001), suggesting that NMDA receptor dependent long-term potentiation (LTP), a classic form of synaptic plasticity extensively studied in the hippocampus (Bliss, T. V., & Collingridge, G. L. (1993), Malenka, R. C. (1994), Tsien, J. Z et al (1996) Lu, W. Y. et al. (2001)), could underlie learning in the amygdala. Indeed, Huang and Kandel (1998) showed that protein kinase A (PKA) dependent postsynaptic LTP, which was partially reliant on NMDA receptors, could be induced in

the LA when stimulating the EC. Furthermore, Rogan et al. (1997) showed that fear conditioning produced similar evoked responses in the lateral amygdala as LTP induction. Later investigations by Nabvi et al. (2014) showed strong supporting evidence that LTP in the amygdala is the underlying cause of fear memory, while long-term depression (LTD) can be used to suppress said memory.

Additional forms of LTP exist in the amygdala. Interestingly, Weisskopf et al (1999) showed that LTP induced by stimulating the IC is NMDA receptor independent. Furthermore, other investigators have shown that cortical glutamatergic projections on to amygdalar interneurons exhibit an NMDA receptor independent form of LTP (Mahanty, N. K., & Sah, P. (1998)), whereas thalamic projections innervating GABAergic interneurons exhibit NMDA receptor dependent LTP (Bauer & LeDoux (2004)). More recent speculation has arisen that these forms of increased glutamatergic drive to interneurons underlie fear extinction learning (Maren, S. (2015)). The important point to keep in mind however, is simply the fact that complex and multiple forms of plasticity are present in the amygdala and these will become a point of consideration later on in the discussion of results.

2.2. Kainate Receptors in the Amygdala

Kainate receptors are non-NMDA-type glutamate receptors with both ionotropic and metabotropic modes of action, composed of tetrameric arrangements of GluK1-5 subunits. (Contractor, A. et al. (2011), Lerma & Marques (2013) Sihra, T. S. et al. (2014)). They are known to be widely expressed in both glutamatergic and GABAergic connections, in pre- and postsynaptic terminals, and they are known to modulate synaptic transmission and plasticity in the amygdala (Li, H. et al. (2001). Braga, M. F. et al. (2003), Braga, M. F. et al. (2009), Aroniadou-Anderjaska, V. et al. (2012), Negrete-Díaz, J. V. et al. (2012)).

In particular, Li, H., & Rogawski, M. A. (1998) showed that GluK1 subunit containing kainate receptors mediate about 25-30% of the total excitatory glutamatergic drive to BLA principal neurons, thereby indicating their important role in the overall activity of the amygdala (see also: Cho et al. (2012)). Aroniadou-Anderjaska, V. et al. (2012) further proved that low concentrations of GluK1 agonist ATPA increases spontaneous excitatory postsynaptic current (sEPSC) and miniature excitatory postsynaptic current (mEPSC)frequency in BLA neurons and can lead to anxiogenic behavior. Additionally, Braga, M. F. et al. (2003) showed that GABA release is modulated by the

activation of presynaptic GluK1 in a concentration-dependent manner, elucidating that GluK1 also plays an important role in GABAergic signaling within the amygdala. Low concentrations of ATPA increased miniature inhibitory postsynaptic current (mIPSC) frequency while the converse happened with larger concentrations. Wu et al. (2007) also showed that activation of GluK1 increases the excitability of GABAergic interneurons, while pyramidal neuron excitability is decreased.

Considering the above results together, it is easily observable that GluK1 kainate receptors shape synaptic activity in the amygdala in complex ways, affecting both glutamatergic and GABAergic signaling. However, it is not known what the net result of these effects are on a synaptic population level. Furthermore, little is known about how kainate receptors modulate synaptic function at different developmental stages, knowing that the expression of various types of kainate receptors (especially GluK1) heavily changes during development (Bettler et al. 1990, Bahn et al. 1994). In the P15 to 22 age range GluK1 and GluK2 kainate receptors are strongly expressed in the amygdala (Braga et al. (2003)), indicating that they may play a significant role in the regulation of amygdalar networks during development.

2.3. The Aims of This Study

Kainate receptors exhibit a variety of effects on synaptic transmission in the amygdala, however, their functions during development of the circuitry are less well understood. The aim of this study was to investigate what the net effect of GluK1 kainate receptors on synaptic population activity and plasticity are in the developing amygdala of P15 to 18 rat pups. To do this, we used extracellular field recordings and studied how postsynaptic glutamatergic responses evoked by afferent stimulation (external capsulae (EC)) change under pharmacological manipulations of GluK1. We also used whole cell voltage clamp to further elucidate the effects of GluK1 on the single cell level. Finally, we investigated if LTP induction is possible in the 2-week-old amygdala and how plasticity is regulated by GluK1.

3. Materials and Methods

3.1. Brief theory of extracellular field potentials and voltage clamp

Extracellular field potentials are generated by charge movement across cell membranes during neuronal population activity. Charged ions (mainly Na^+, K^+, Ca^{2+} and Cl^-) moving across cell membranes during synaptic activity create transmembrane currents that can be measured from the extracellular space (Leung, L. W. S. (1990)). To exemplify this, let's consider a single pyramidal neuron, shown also in Figure 1. Activation of excitatory synaptic contacts in the dendritic tree cause an influx of positive ions in to the postsynaptic compartments. This will cause a positive current to flow in to the cell, momentarily leaving the extracellular negatively charged. If we were to place an electrode in the extracellular space near the dendritic tree during this conductive event, we would measure a negative voltage deflection (voltage being the product of current and resistance). This point in the extracellular space then becomes a current sink.

The positive current flowing in to the postsynaptic compartments does not simply remain there but continues to flow through the resistive intracellular fluid towards the soma. Kirchhoff's first law states that at any point in an electrical circuit, current inflow and outflow must sum to zero. This law is derived from the fundamental physical principle of charge conservation, which states that the amount of positive and negative charges in an isolated system is always conserved. This means that any charge flowing in to a given volume of space must be matched by an outflow of charge from that volume of space. Accordingly, positive current that rushed in at the distal dendrites, exits at the soma, creating a current source. Relative to the current sink - the point in the extracellular space that during this conductive event becomes negatively charged - the current source will be positively charged. Thus, if we were to place an electrode in the extracellular medium near the soma during this conductive event, we would observe a positive voltage deflection.

The outflowing current at the current source then flows back through the resistive extracellular medium to the current sink, forming a closed electrical circuit. Kirchhoff's second law states that that the sum of voltages in a closed loop in an electrical circuit must equal zero. This is derived not only from the principle of charge conservation is also related to even more fundamental principle of conservation of energy, without which physical phenomena as we know them would not be

possible. Hence, these considerations of Kirchhoff's laws are not arbitrary but fundamental to understanding how electrical phenomena in the extracellular space take place.

It should also be kept in mind that when discussing extracellular field potentials, we are always talking about the activity of hundreds or thousands of neurons, depending on where we place the recording electrode. Thus, the voltage deflections measured are the combinatory current sinks and sources generated by the simultaneous activity of many neurons. Figure 1C exemplifies current source and sink densities generated by a laminar array of neurons and this is commonly how extracellular field potentials measured from the hippocampus are theorized to be generated (Holsheimer, J. (1987)). However, due to the complex neuronal architecture of the amygdala, the neurons contributing to the measured signal become much more difficult to discern. This will become a point of consideration later on in the discussion.

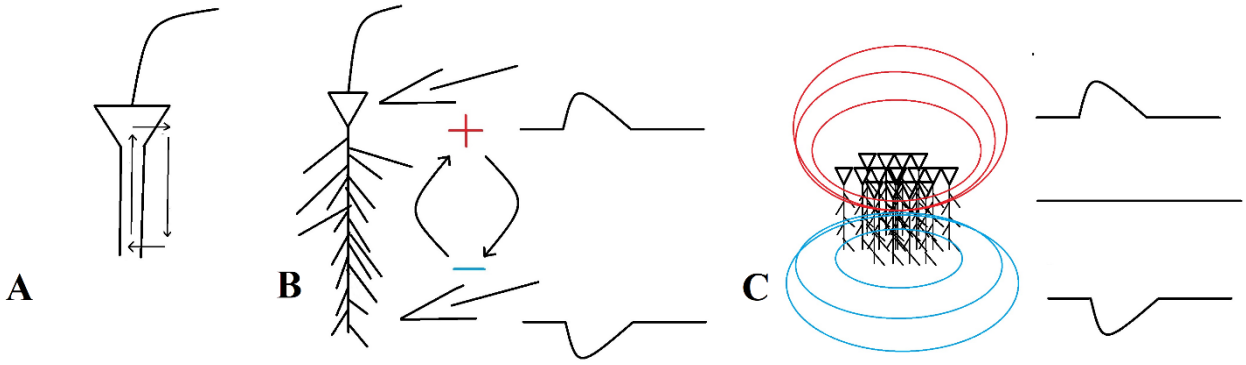


Figure 1.

A) Current loop formed in a pyramidal neuron when excitation arrives at the distal dendrites.

B) A more macroscopic view of the pyramidal neuron and the voltage deflection observed from the extracellular space. Placing the measurement electrode near the apical dendrites (current sink) during a conductive event will result in the measurement of a negative voltage deflection. Conversely, measuring from the somatic area (current source) will result in the measurement of a positive voltage deflection.

C) Current sink and source densities created by a laminar array of neurons.

In whole cell voltage clamp, currents flowing through the entire cell can be recorded, while controlling the membrane potential to a desired level. A patch glass microelectrode is placed on the

membrane of a cell and suction is applied. This allows the microelectrode to make tight contact with the membrane and form a giga-ohm seal or “gigaseal”. To enter the whole cell configuration, additional suction is applied to rupture the membrane, thus allowing the microelectrode to access the intracellular compartment

To keep the membrane potential constant, the measurement system has to apply compensatory current when the membrane potential changes. Once the electrode detects a change in membrane potential due to ionic currents flowing across the membrane, this is fed to a feedback amplifier along with a voltage command signal. The command signal expresses the desired membrane potential which the experimenter wants to hold the cell at. If the changed membrane potential differs from the command signal, an error signal is sent through a current generator, which then sends a current back down to the cell to return the membrane potential back to the command value. It’s important to note that this injected current is the current that the voltage clamp system measures and that this injected current is equal to ionic currents mediated by ion channels on the cell membrane. This is because in order to keep the membrane potential constant, the injected current has to exactly match all ionic currents in the cell that attempt to change the membrane potential from the command value. As an example, let’s say that we are clamping the membrane potential at -70 mV and at a certain point in time, cations flow in to the cell, causing membrane depolarization to -60 mV. The voltage clamp system measures this depolarization and injects a negative current in order to bring the membrane potential back down to – 70 mV. Hence, currents caused by cations that bring positive charge in to the cell, will be measured as negative current deflection, since the injected current is negative. Similarly, hyperpolarizing currents will be seen as positive current deflections, since the injected current to counteract membrane hyperpolarization from the command value will be positive.

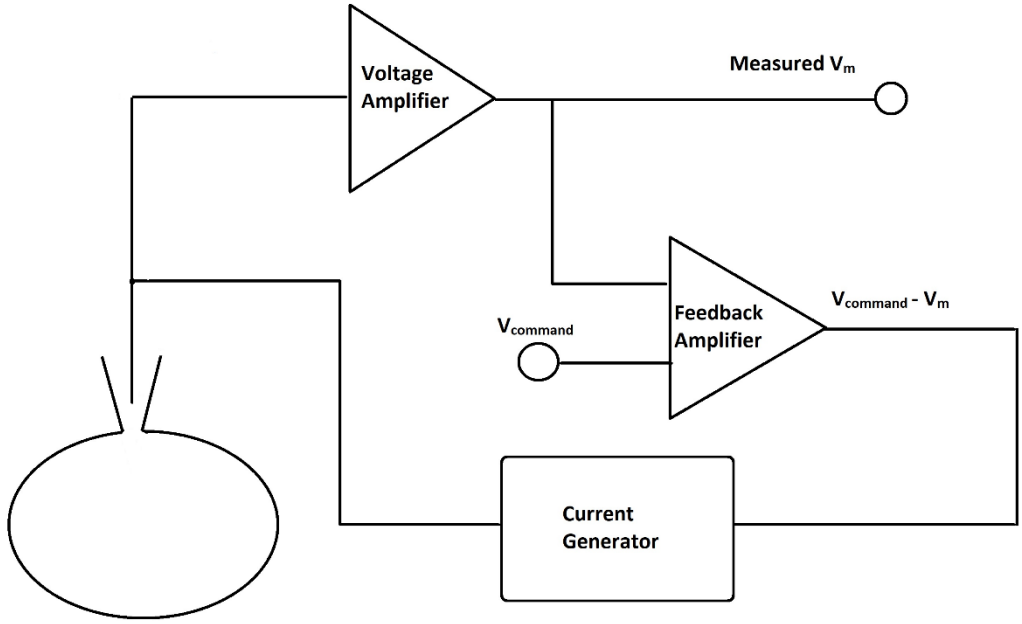


Figure 2. A simplified schematic of single electrode whole cell voltage clamp. The microelectrode measures voltage changes across the membrane (V_m). This amplified signal travels to the feedback amplifier which compares V_m to the experimentally set holding voltage ($V_{command}$). If there is a difference between the two an error signal is sent to the current generator which produces a current that is then injected to the cell in order to keep the membrane potential at $V_{command}$.

3.2. Experimental procedures

Animals used and ethical statement. Wistar Han rat pups aged P15-P18, both sexes, were used in this study. The total number of animals in these experimental results is 25, one animal used per experiment. To minimize suffering during the decapitation procedure, animals are first anesthetized with isoflurane. Loss of consciousness is ensured by a lack of reaction to pain by strongly squeezing the tail with tweezers. Decapitation is done quickly to make sure the animal does not wake up or feel pain from the procedure. All experiments with animals were done in accordance with the University of Helsinki Animal Welfare Guidelines.

Reagents. All pharmacological compounds used in this study were supplied by Tocris. The pharmacological agents used were selective GluK1 antagonist ACET (200nM), selective GluK1 agonist ATPA (1 µM), AMPA/kainate receptor antagonist 6-Cyano-7-nitroquinoxaline-2,3-dione

(CNQX) (20 µM), sodium channel antagonist tetrodotoxin (TTX) (1 µM) and $GABA_A$- receptor antagonist picrotoxin (PiTX) (100 µM).

For electrophysiological recordings P15 to P18 rat pups were anesthetized with isoflurane and quickly decapitated. The brain was extracted and immediately placed in carbogenated (95% O_2/5%CO_2) ice-cold modified artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $MgSO_4$ 11, $NaHCO_3$ 26, D-glucose 15, Ca_2Cl 1. The cerebellum and a small part of the prefrontal cortex were trimmed off, the brain glued to a stage and transferred to a vibratome (Leica VT 1200S) to obtain 400 µm thick brain slices. Slices containing the amygdala were placed in to a slice holder in 37ºC heat bath, with the holder containing standard ACSF with 2 mM Ca_2Cl and 1 mM $MgSO_4$ Slices were incubated in the heat bath for 30-40 minutes and in room temperature for an additional 30 minutes.

For extracellular field recordings the brain slices were transferred to a carbogenated and preheated interface recording chamber (32-33ºC) and were perfused with prewarmed (34ºC) ACSF with the speed of 1.25 ml/minute. To obtain stable local field potential (fEPSP) from the developing amygdalar circuit, the stimulation and recording electrodes had to be placed rather close to each other. Stimulation electrode was placed in to the EC containing cortical projection axon, very near the apical part of the amygdala, and the recording electrode was placed near the top of the lateral amygdala (LA). Recording electrode glass micropipette (Harvard Apparatus 1.5 OD x 0.56 ID x 75L mm) resistance was around 3-5 MΩ and was filled with ACSF. In order to obtain stable recordings, slices were allowed to sit in the recording chamber for 15 – 30 minutes before the experiment was started. This helped attenuate drift in amplitude and/or slope. Recordings consisted of 15 minutes of baseline, after which a drug was added or LTP was induced using 100Hz/s stimulation, depending the experiment. During the experiments using picrotoxin, fEPSPs often became polysynaptic which made the measurement of fEPSP amplitudes difficult. If the amplitude did not remain stable after bath application of picrotoxin, the recording was disqualified. fEPSPs were amplified (Axon Instruments AxoPatch 200B, 100x gain), lowpass filtered (5 kHz), A/D converted (Axon Instruments Digidata 1322 16-bit data acquisition system, 20 kHz sampling frequency) and gathered/analyzed online and offline with WinLTP version 1.11.

For whole cell voltage clamp experiments slice preparation was done using a sucrose-based dissection solution containing (in mM): NaCl 87, $NaHCO_3$ 25, KCl 2.5, NaH_2PO_4 1.25, $MgSO_4$ 7, Ca_2Cl 0.5, D-glucose 25, sucrose 50. Slices were placed in a submerged heated (37ºC, Luigis and Neumann Badcontroller V) recording chamber and perfused with standard ACSF at the speed of 1.25 ml/minute. LA neurons were patched using 5-7 MΩ glass electrode micropipettes, filled with a

potassium based low chloride filling solution containing (in mM): K-gluconate 135, HEPES 10, KCl 2, $CaOH_2$ 2, EGTA 2, Mg-ATP 4, Na-GTP 0.5. After obtaining a strong gigaseal, the cell membrane was ruptured to enter whole-cell configuration. The membrane potential was clamped at -70 mV, and the cell was allowed to equilibrate with the filling solution for 10 minutes. Afterwards the membrane potential was slowly raised to 0 mV, the reversal potential of glutamatergic currents, in order to only measure spontaneous inhibitory postsynaptic currents (sIPSC). The recording was once again allowed to stabilize for 5-10 minutes prior to baseline measurement. After baseline recordings (10 min), 1 µM ATPA was perfused in to the bath (15-20 min) and a rewash period was carried out for 20 minutes. The recorded signal was amplified (Axon Instruments Multiclamp 700B), lowpass filtered (5 kHz), A/D converted (Axon Instruments digidata 1322 16-bit data acquisition system, 20 kHz sampling frequency) and gathered with WinLTP version 1.11. sIPSC frequency analysis was done manually using Minianalysis program 6.0.3. sIPSCs were identified in the analysis as outward currents with typical kinetics, that were at least 3 times the amplitude of the baseline level of noise.

Statistical analysis. Averages and the standard error of mean (S.E.M.)s of the analyzed properties of synaptic events were calculated for baseline and drug/LTP condition, for repeated experiments. The latter averages were calculated 15 minutes after drug application and 50 minutes after 100Hz/s stimulation, respectively. When comparing two conditions within an experiment, two tailed t-test p-values were calculated from raw data values. One-way ANOVA was used for statistical comparison when comparing between experimental groups. These are the statistical values presented throughout this paper. For graphical representation of measurements, fEPSP amplitude and slope were normalized to baseline, and these normalized values were averaged across measurements. The graphs represent averaged values, with error bars corresponding to S.E.M values. Statistical significance is denoted with * ($p < 0.05$)

4. Results

4.2. Pharmacological characterization of the amygdalar fEPSP

First, we wanted to confirm that the synaptic origin of the extracellular signal, recorded from the lateral amygdala in response to EC stimulation in brain slices from juvenile (P15-18) rat pups. The measured signal consisted of two negative deflecting voltage peaks (*First voltage peak amplitude:* -

-0.33 ± 0.03 mV, slope: -0.62 ± 0.04 mV/ms - Second voltage peak amplitude: -0.75 ± 0.02 mV, slope: -0.39 ± 0.02 mV/ms). To see if either of these were mediated by postsynaptic ionotropic glutamate receptors, we used CNQX (1 µM) to block postsynaptic AMPA/kainate receptors. This caused the second, larger voltage peak to disappear from the signal (*Amplitude: 0.06 ± 0.02 mV, Slope: 0.14 ± 0.02 mV/ms*), (Figure 3B, middle trace), indicating that the latter part of the signal reflected a field excitatory postsynaptic potential (fEPSP) (n = 5). Next, we used sodium channel blocker TTX (1 µM) in conjunction with CNQX (20 µM) to see if this would remove the remaining voltage peak (*Amplitude: -0.31 ± 0.01 mV – Slope: -0.41 ± 0.05 mV/ms*). Indeed, in the presence of TTX, the remaining part of the signal was eliminated, leaving only the stimulus artefact (Figure 3B, bottom trace) (n = 5). This indicated that this element of the signal was a presynaptic fiber volley, mediated by activation of axonal or presynaptic voltage-gated sodium channels.

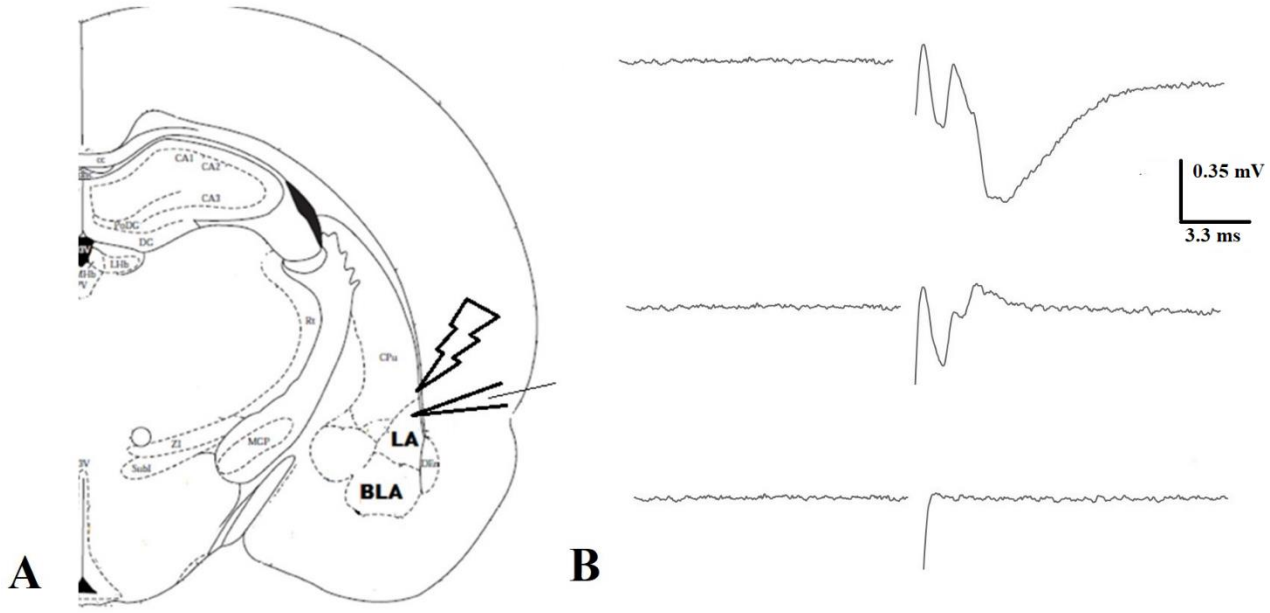


Figure 3.

A) Coronal rat brain slice indicating the electrode positions for fEPSP recordings. Stimulation electrode is placed on the external capsule, near the apical part of the amygdala. Recording electrode is placed in the lateral amygdala (LA).

B) Traces showing the basic pharmacological characterization of the fEPSP obtained from the LA. Top trace indicates baseline recording, showing voltage gated sodium channel mediated presynaptic fiber volley as a small negative deflection, followed by a larger negative deflection indicating postsynaptic sodium conductance through AMPA/KA receptors. Middle trace shows what happens to the fEPSP when AMPA/KA receptor antagonist CNQX (20µM) is used. The postsynaptic AMPA receptor mediated element is removed and only the presynaptic fiber volley remains.

Bottom trace shows the signal while perfusing CNQX and sodium channel blocker TTX (1µM) in to the brain slice. Only the stimulus artefact remains.

4.3. ACET had no effect on the fEPSP

After having confirmed pharmacologically that a postsynaptic glutamatergic field response (fEPSP) could be reliably evoked in response to EC stimulation in the juvenile amygdala, we next sought to test if GluK1 kainate receptor blocker ACET (200 nM) had any effects on the fEPSP. We observed no significant changes in fEPSP slope (*fEPSP slope: 102.2 ± 3.5 % of baseline, n = 3, p = 0.61, Students two-tailed, paired t-test*) or amplitude (*fEPSP amplitude: 109 ± 5.9% of baseline, n = 3, p = 0.17, Students two-tailed, paired t-test*) in response to application of ACET (Figure 4).

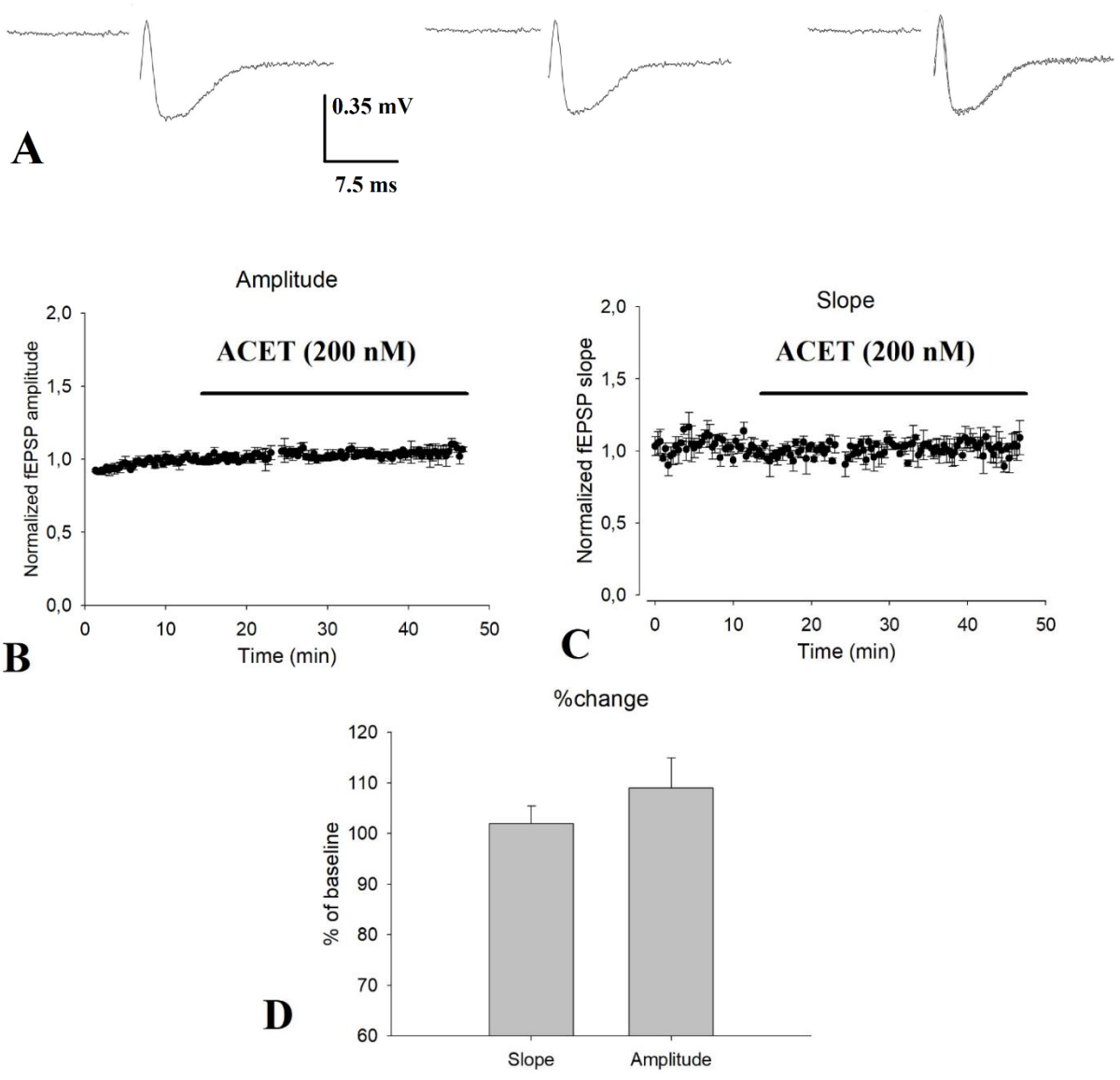


Figure 4.

A) Example fEPSP traces from left to right: baseline, ACET, superimposed.

B) A time-course plot representing the effects of ACET on fEPSP amplitude (n = 3).

C) A time-course plot representing the effects of ACET on fEPSP slope (n = 3).

D) Histogram showing the % of baseline of both slope and amplitude.

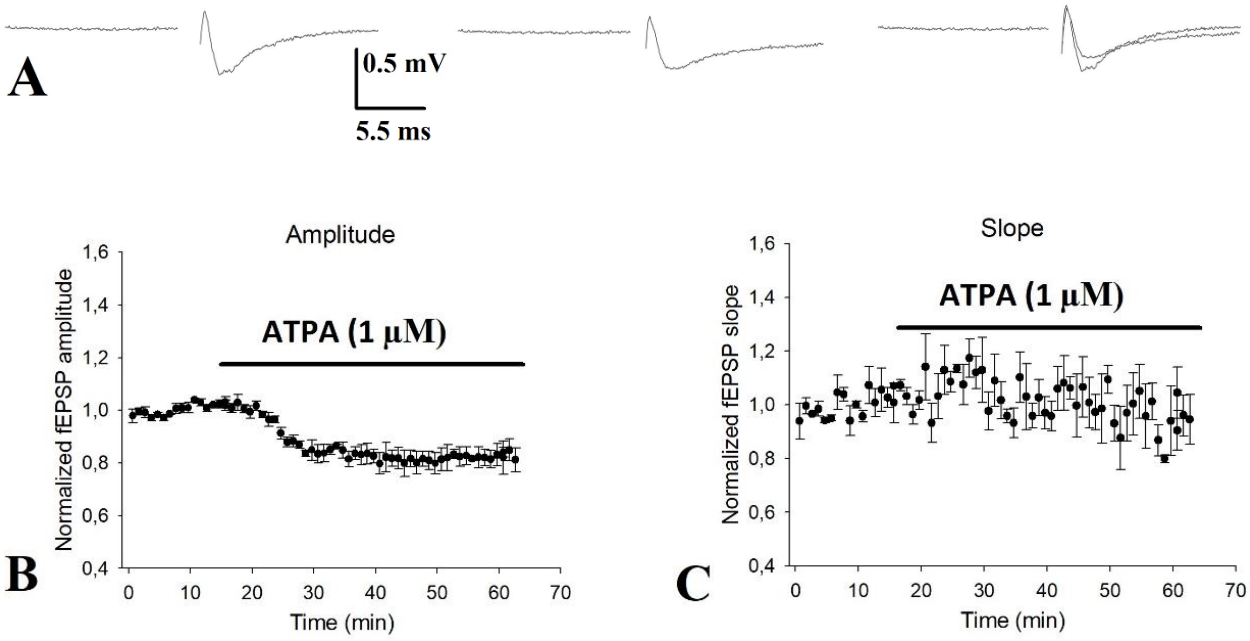
4.4. Activation of GluK1 with ATPA increases GABAergic inhibition in the amygdala

Seeing that blocking GluK1 kainate receptors with ACET produced no significant effect, we wanted to see whether the activation of GluK1 with ATPA (1 µM), a selective GluK1 agonist, would affect the fEPSP. Interestingly, the addition of ATPA reduced the peak amplitude of the fEPSP (*fEPSP amplitude: 82.6 ± 3.5 % of baseline, n = 3, p = 0.001, Students two-tailed, paired t-test*) but seemed to have no effect on the slope (*fEPSP slope: 95.9 ± 7.8% of baseline, p = 0.52, Students two-tailed, paired t-test*) (Figure 5A, B, C, D and E).

This suggested to us that perhaps the activation of GluK1 is increasing GABAergic inhibition targeted at the synaptic population. To confirm this hypothesis, we tested whether or not ATPA would have a similar effect on the fEPSP amplitude, while blocking $GABA_A$ receptors with $GABA_A$ receptor antagonist picrotoxin (PiTX) (100 µM). Picrotoxin changed the shape and increased the amplitude (*fEPSP amplitude: 134.6 ± 4.5 % of baseline, n = 3, p = 0.02, Students two-tailed, paired t-test*) and the slope (*fEPSP slope: 145 ± 12.8 % of baseline, n = 3, p = 0.02, Students two-tailed, paired t-test)* of the fEPSP, also causing an increase in polysynaptic activity. Adding ATPA to the perfusion in the presence of PiTX caused a mild decrease in both the slope (*fEPSP slope: 135.3 ± 13.6 % of baseline, n = 3, p = 0.05*) and amplitude (*fEPSP amplitude: 126.8 ± 3.9 % of baseline, n = 3, p = 0.02, Students two-tailed, paired t-test*), as compared to the level in PiTX (Figure 5D, E, F, G, H and I). However, the effect of ATPA on fEPSP amplitude in the presence of PiTX was significantly smaller as compared to its effect under control conditions (*fEPSP amplitude (PiTX + ATPA): 94.2 ± 1,4 % of 10 min stabilized PiTX region - fEPSP amplitude (ATPA): 82.6 ± 3.5 % of baseline, F (1,4) = 18.731; p = 0.012, One-way ANOVA*). Additionally, drift or slice deterioration due to extensive picrotoxin use as the cause of the

amplitude reduction in the picrotoxin + ATPA experiments cannot be ruled out either with such a small sample size.

To further ensure that inhibitory signaling is increased in the amygdala due to the activation of GluK1, we measured the effects of ATPA on sIPSC frequency in LA neurons using whole-cell voltage clamp. ATPA significantly increased sIPSC frequency in the amygdala (*sIPSC frequency:3.88 ± 0.98x the baseline, n = 9, p = 0.007, Students two-tailed, paired t-test*) but had no significant effects on sIPSC amplitude (*sIPSC amplitude: 1.09 ± 0,04x baseline, n = 9, p = 0.32, Students two-tailed, paired t-test)* (Figure 6 A, B and C). These results suggested to us that the activation of GluK1 causes an increase in spontaneous inhibition in the amygdala, thereby causing the reduction in fEPSP amplitude seen in the field potential recordings.



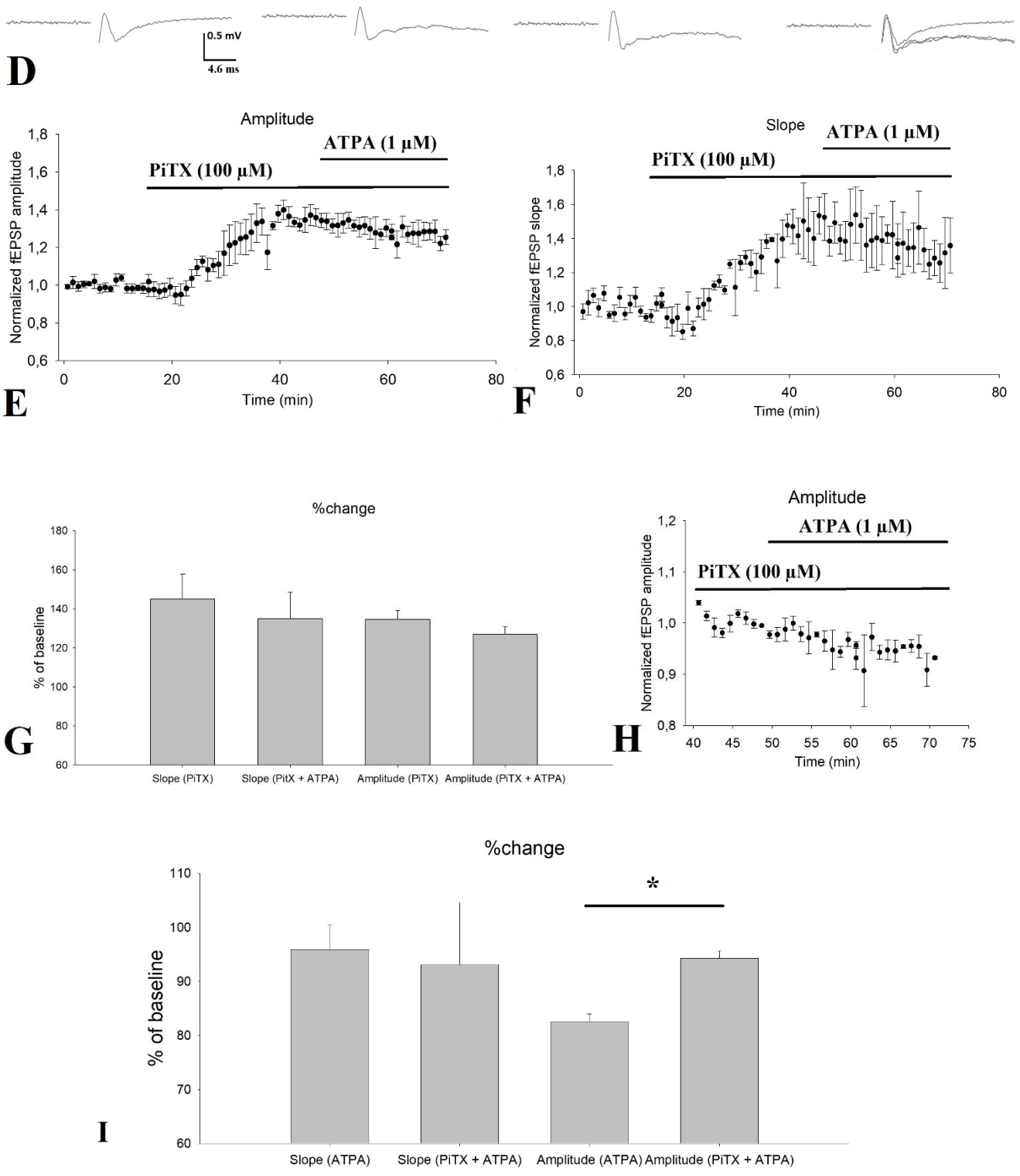


Figure 5.

A) Example fEPSP traces from left to right: baseline, ATPA (1 µM), superimposed.

B) Effects of ATPA on fEPSP amplitude (n = 3). Activation of GluK1 reduces fEPSP amplitude.

C) Effects of ATPA of fEPSP slope (n = 3). Activation of GluK1 has seemingly no effects on fEPSP slope.

D) Example fEPSP traces from left to right: baseline, picrotoxin (100 µM), picrotoxin (100 µM) + ATPA (1 µM), superimposed.

E) Effects of picrotoxin and ATPA on fEPSP amplitude (n = 3). Picrotoxin increases fEPSP amplitude and attenuates the effects of ATPA.

F) Effects of picrotoxin and ATPA on fEPSP slope (n = 3).

G) Pooled data showing the level of fEPSP amplitude and slope in the presence of drug perfusion(s). The data is expressed as % change compared to the baseline in the beginning of the experiment.

H) A closer look at the fEPSP amplitude during the combined perfusion of ATPA and picrotoxin. Re-normalization of the data to the level in PiTX shows that activation of GluK1 in conjunction $GABA_A$ receptor blockade still causes a mild reduction in fEPSP amplitude

I) Histogram depicting the effect of ATPA on fEPSP slopes and amplitudes under control conditions (ATPA) and in the presence of PiTX (ATPA + PiTX). The data is expressed as the % change compared to the level 0-10 min before ATPA application. Asterisk denotes a statistically significant difference in amplitudes (ATPA vs. PiTX + ATPA) (p = 0.012, One-way ANOVA).

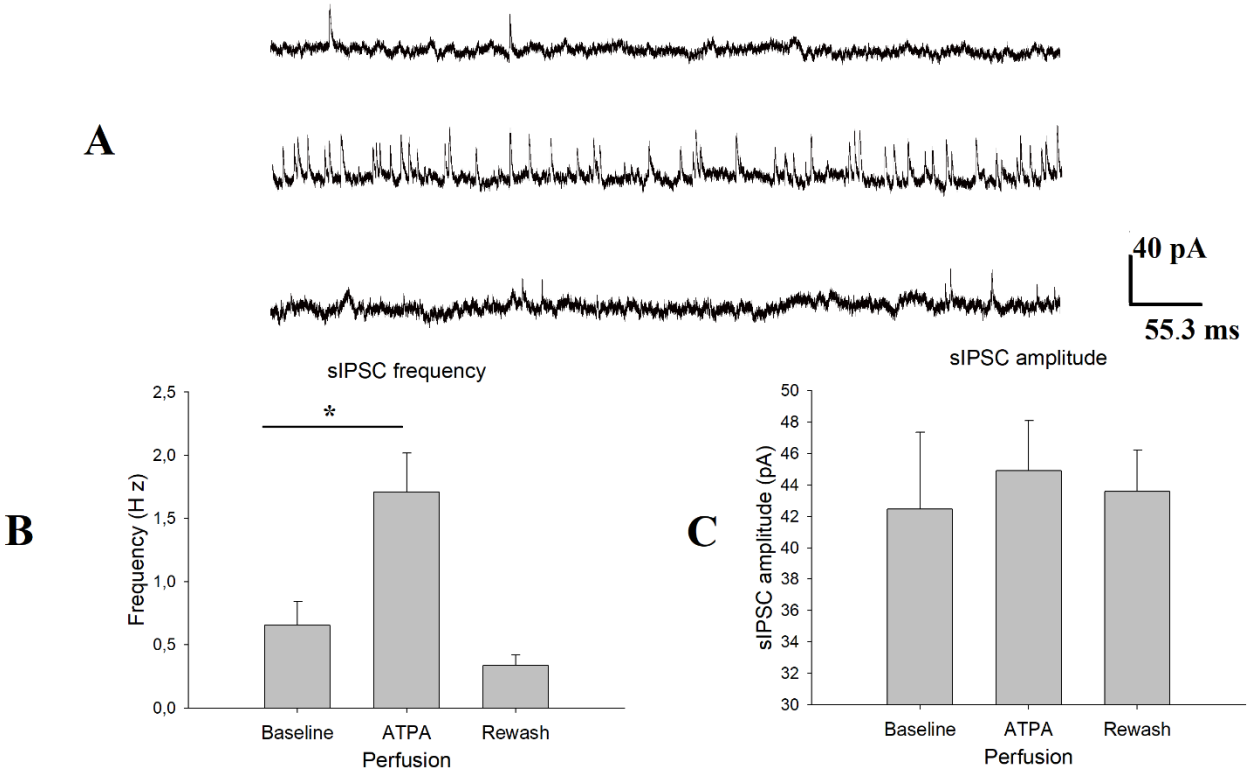


Figure 6.

A) Example traces from sIPSC recordings in the amygdala. Top to bottom: baseline, ATPA (1 µM), rewash.

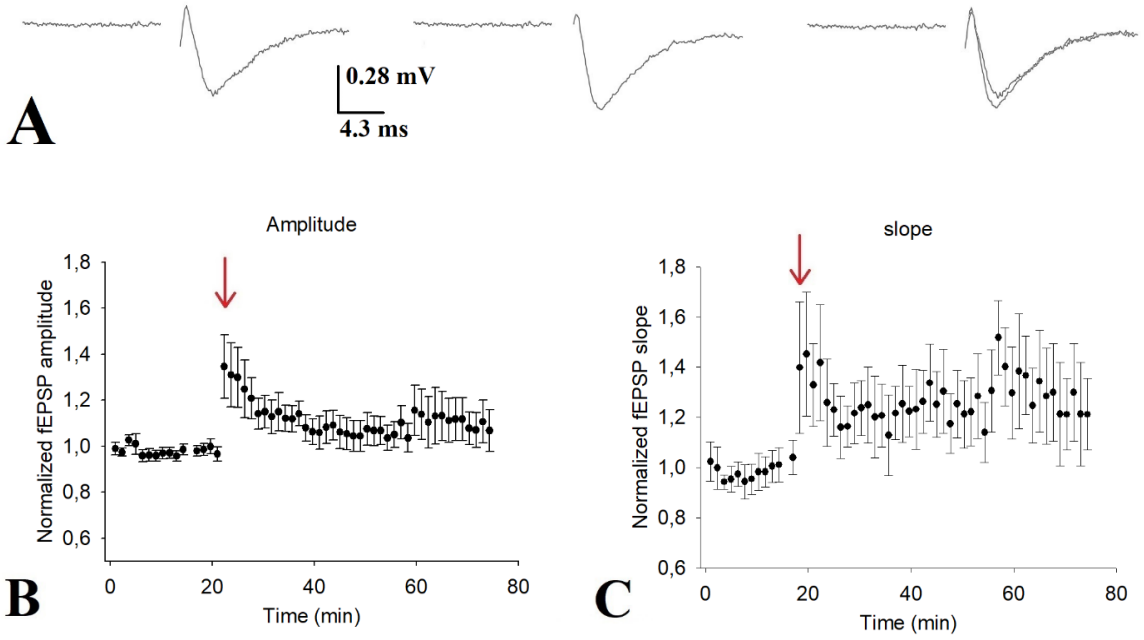
B) Histogram showing the effects of ATPA on sIPSC frequency. Activation of GluK1 heavily increases sIPSC frequency (n = 9). Asterisk denotes statistical significance between baseline and ATPA (p = 0.007, Student's two-tailed, paired t-test).

C) Histogram showing the effects of ATPA on sIPSC amplitude.

4.5. Activation of GluK1 attenuates LTP induction

Since plasticity in the amygdala is speculated as being the underlying mechanism for fear learning, we wanted to see if LTP induction was modulated by GluK1 activation. Using a 100Hz/s stimulation protocol a mild long term potentiation of fEPSPs could be induced in the amygdala (*fEPSP slope: 131 ± 16 % of baseline, n = 7, p = 0.16, Students two-tailed, paired t-test - fEPSP amplitude: 109 ± 8.6 % of baseline, n = 7, p = 0.37, Students two-tailed, paired t-test*) (Figure 7A, B, C, D and E). While there is an observable effect of the stimulation, this effect was not statistically significant.

The activation of GluK1 by ATPA (1 µM) produced a similar reduction in amplitude as observed above and significantly attenuated LTP induction and, in fact, produced LTD when comparing the baseline to 50 minutes after 100Hz/s stimulation (*fEPSP slope: 88.8 ± 12.9 % of baseline, n = 4, p = 0.47, Students two-tailed, paired t-test – fEPSP amplitude: 86.3 ± 3.2 % of baseline, n = 4, p = 0.01, Students two-tailed, paired t-test*). (Figure 7D, E, F and G). Comparing the level of synaptic potentiation, induced by 100Hz/s stimulation in the control conditions vs in the presence of ATPA gave statistically significant results (*Slope ANOVA: F (1,9) = 7.138, p = 0.028 – Amplitude ANOVA: F (1,9) = 6.808 p = 0.031*). This suggests that the increased inhibitory signaling in the amygdala due to the activation of GluK1 is strong enough to attenuate plasticity.



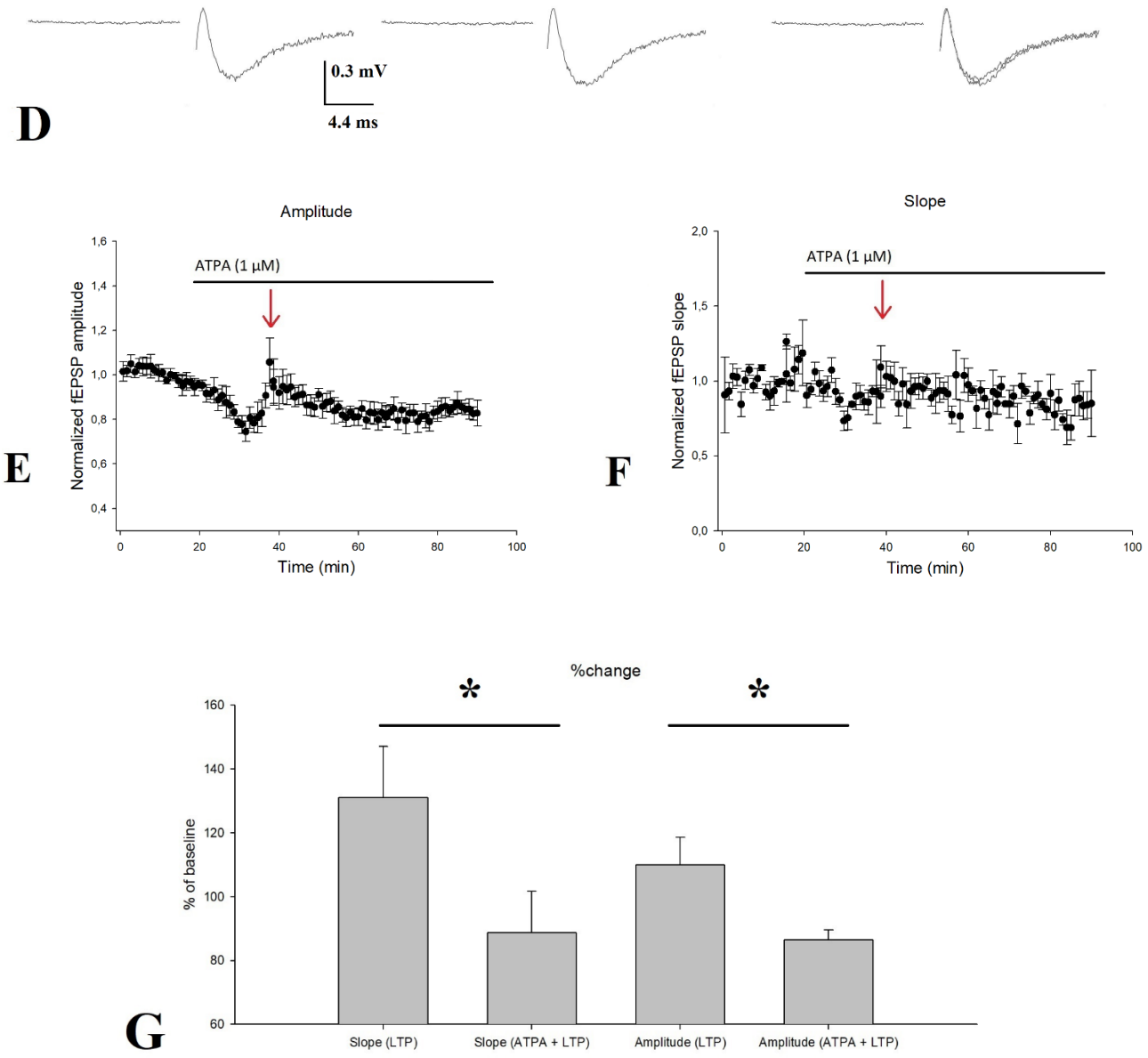


Figure 7.

A) Example fEPSP traces from left to right: baseline, 60 min after 100Hz/s stimulation, superimposed.

B) A time course plot illustrating the effects of 100Hz/s stimulation (marked with a red arrow) on fEPSP amplitude and slope (C) (n = 7).

D) Example fEPSP traces from left to right: baseline, ATPA, ATPA + 60 min after 100Hz/s stimulation

E) Effects of 100Hz/s stimulation on fEPSP amplitude, in conjunction with GluK1 kainate receptor activation (n = 4). The addition of ATPA causes a similar reduction in amplitude as shown in Figure 5B and blocks LTP induction.

F) Effects of 100Hz/s stimulation on fEPSP slope (n = 4), in conjunction with GluK1 kainate receptor activation. LTP induction is blocked due to the effects of ATPA.

G) Pooled data comparing the effect of 100Hz/s stimulation on fEPSP slope and amplitude, under control conditions (n = 7) and in the presence of ATPA (n = 4). Asterisks denotes statistical significance in both slope ($p = 0.028$) and amplitude ($p = 0.031$) (One-way ANOVA).

5. Discussion

5.1. Methodological considerations

Here, we were able to show that extracellular field potential recordings can provide information about how synaptic activity and plasticity are shaped by GluK1 subunit containing kainate receptors in the juvenile rat amygdala. However, it must be noted that due to the nature of these recordings, accurate mechanistic explanations for the effects remain elusive. The fEPSP shows the aftermath of various neuronal conductance events from the extracellular space. The sources and sinks that contribute to local field potentials are numerous beyond synaptic activity – fast action potentials, calcium spikes, intrinsic currents, varying afterhyperpolarizations of different types of neurons, gap junctions and ephaptic coupling (Buzsáki et al. 2016). Taking measurements from a developing synaptic population and knowing that the amygdala is intrinsically much more disorganized in its neuronal architecture, provided a challenge in discerning whether or not reliable postsynaptic field excitatory postsynaptic potentials (fEPSPs) could be obtained. Accordingly, we first sought to pharmacologically characterize the obtained extracellular signal, in order to be certain that we were measuring postsynaptic events. Having confirmed that the extracellular signal was indeed a glutamatergic fEPSP, we could then move on to study how inhibition and activation of GluK1 affected the synaptic population activity and plasticity.

It's also important to note that wherever the measurement electrode is placed in the tissue, the resulting fEPSP is composed of all local neuronal and synaptic activity, including both excitatory and inhibitory signaling. In conjunction with this neuronal geometry and synaptic distribution has a strong effect on what kind of local field potential one can measure (Rall, W. (1962), Lindén et al. (2011), Buzsáki et al. (2016)). Combined with the considerations above, it's easy to see how synaptic signaling can be masked by a plethora of effects when measuring extracellular signals. Hence, it would always be pertinent to combine extracellular recordings with intracellular ones, in order to truly unmask the effects any given treatment may have on synaptic signaling and plasticity.

A brief mention about statistics is in order. Most of the measurements done here had a very low n-number. Despite the effects of a given treatment being very consistent, with low variability, in hindsight it would have been better to add a few more experiments to each test. One must also take note of the role of significance tests in scientific research. Statistical significance does not always equal to biological significance and vice versa. Hence, it is important to report both the effects and the statistical results as separate entities, and not outright dismiss a result if a clear effect can be seen but no statistical significance is obtained (Carver, R. (1978), Johnson, D. H. (1999), McShane, B. B. et al. (2017), Gigerenzer, G. (2018)).

5.2. Blocking GluK1 had no effect on fEPSP

Blocking GluK1 with ACET (200nM) had no significant effects on the fEPSP, evoked by EC stimulation in the lateral amygdala. However, this does not rule out the possibility that there may exist subtler effects on the neuronal level that are masked by the methodology used here. Indeed, Braga et al. (2009) have observed in slightly older animals (P18 to 24) that blocking GluK1 with topiramate leads to complex alterations of inhibitory and excitatory currents targeted to LA principal neurons. Blocking GluK1 seemed to reduce excitatory currents from glutamatergic afferents while simultaneously leading to the blocking of the suppression of inhibitory GABAergic signaling via inactivation of presynaptic GluK1 receptors on interneuron synaptic terminals - overall driving the amygdalar network towards increased inhibition. One must take note of the fact however, that topiramate is a non-selective antagonist for GluK1 and also attenuates voltage gated sodium channels (Zona et al (1997)), inhibits carbonic anhydrase isoenzymes (Dodgson et al. (2000)) and acts as an allosteric modulator of $GABA_A$ receptors (Simeone, T. A. et al (2006), Simeone, T. A et al. (2011). These effects may also contribute to their results, especially the $GABA_A$ receptor modulation. Aroniadou-Anderjaska, V. et al. (2012) et al. showed that GluK1 antagonist UBP302 (25 µM) can reduce mEPSC frequency as well as reduce the amplitude of evoked field potentials and enhance their paired-pulse ratio. However, this was done in much older rats (35 to 50 day old). Since UBP 302 is a strongly selective GluK1 antagonist, it may be that the developmental stage of the amygdalar network contributes to these results. Similar results were produced by Cho et al. (2012), whom observed that UBP 302 reduces EPSC amplitude by roughly 20 % in 3- to 4-week-old rats.

A future step for research could be to study the effects of ACET on EPSCs and IPSCs on the single cell level, to uncover whether or not some kind of refined dynamics exist and how these dynamics may change during development. Perhaps blocking GluK1 with ACET may also have some effects on LTP that were not explored in the present study.

5.3. Activation of GluK1 with ATPA increases GABAergic inhibition in the amygdala

Here, we were able to show that the activation of GluK1 kainate receptors increase GABAergic inhibition within the developing amygdala on both the synaptic population and single-cell levels. This is well in accordance with previous results obtained by other groups (Braga, M. F. et al. (2003), Aroniadou-Anderjaska, V. et al. (2007) Braga, M. F. et al. (2009)). Additionally, similar effects of GluK1 on GABA release and the activation of interneurons has been well documented in the hippocampus (Clarke, V. R., (1997). Cossart, R. et al. (1998), Cossart, R. et al. (2001), Jiang, L. et al. (2001), Jiang, L. et al. (2015)). Conversely, Li et al. (2001) showed that 20 µM ATPA could induce strong potentiation of excitatory postsynaptic potentials EPSP. Considering the high concentration used in conjunction with the results of Braga et al. (2003), whom show that high concentrations of ATPA (10 µM) cause a decrease in GABAergic inhibitory transmission targeted at BLA principal neurons, it seems likely that this is the reason why Li et al. (2001) observe a strong potentiation of the EPSP. We used a much lower concentration of ATPA (1 µM, specific concentration for GluK1 agonism), which in accordance with Braga et al. (2003) produces an increase in GABAergic transmission in the amygdala.

Interestingly, our results further suggested that the increase in inhibitory signaling due to the activation of GluK1 is sufficient to block LTP induction in the lateral amygdala altogether. In accordance with the whole-cell voltage clamp measurements presented here, it does seem reasonable that such a heavy increase in inhibitory signaling targeted at the LA principal neurons would attenuate learning paradigms such as LTP. The fact that LA principal neurons are known to be heavily innervated by both parvalbumin and somatostatin interneurons (Muller, J. F. et al. (2006), Muller, J. F. et al. (2007)) and both of these interneuron types form local microcircuits that control fear learning (Ehrlich, I. (2009), Lucas, E. K. et al. (2016)), adds more confidence to the results obtained here. We hypothesize that the increased inhibition targeted at amygdala neurons

during GluK1 activation prevents strong membrane depolarization and thus NMDA receptor activation.

When considering LTP induction in the control case, one must take note of the methodological limitations of the experiments done here once again. As discussed in the introduction, multiple forms of LTP exist in the amygdala that cause potentiation in both glutamatergic and GABAergic synapses (Huang & Kandel (1998), Mahanty, N. K., & Sah, P. (1998), Weisskopf et al (1999), Bauer & LeDoux (2004), Maren, S. (2015)). When using a strong stimulation protocol, such as the 100Hz/s stimulation used here, a complex set of plasticity may occur within the synaptic population. Although masked by the limitations of extracellular field potentials, as discussed in 5.1, some speculation about the mechanisms and in particular, regarding the role of GluK1 in LTP induction can be made. Braga, M. F. et al. (2003) showed in P15 to 21 male Sprague Dawley rats GABAergic transmission targeted at pyramidal neurons in the amygdala is mediated by GluK1 agonists in a concentration-dependent manner. Low concentrations of both ATPA and glutamate increased GABA release while high concentrations of these compounds had the opposite effect. Additionally, extracellular endogenous glutamate also increased the failure rates of evoked IPSCs (eIPSCs) but not sIPSCs. Taken together, they propose the idea that during strong stimulation, extracellular concentrations of glutamate become high enough to attenuate GABAergic transmission by acting on GluK1 kainate receptors on the presynaptic terminals of interneurons. Conversely, spontaneous activity would only produce low concentrations of extracellular glutamate, causing in increase in GABAergic transmission via the activation of somatodendritic GluK1 kainate receptors. Considering that in our field potential recordings we were able to observe a mild potentiation in response to strong afferent (EC) stimulation, in rat pups of a similar age, the mechanism proposed by Braga et al. (2003) could be at work here as well. While strong EC stimulation is activating both GABAergic and glutamatergic synapses, it seems that the net effect of this activity leads to LTP, perhaps permitted or facilitated by reduced GABAergic transmission via GluK1.

A further approach could be to see how LTP is induced as such and under GluK1 manipulation on the single-cell level with whole cell voltage clamp. A simple pairing protocol coupling afferent stimulation to a brief membrane depolarization could potentially induce LTP and ATPA could easily be perfused in to the slice in order to see its' effects. Additionally, if we wished to stick with field potential recordings, the ATPA + LTP experiments should also be carried out while blocking $GABA_A$ receptors with picrotoxin. This would show more clearly if a causal relationship exists between the increased inhibition caused by GluK1 activation and the attenuation of LTP induction.

It would also be interesting to observe how these dynamics between kainate receptors and GABAergic inhibitory signaling change during development, since this study is only limited to P15-18-time window.

Some care must also be taken in interpreting the data where picrotoxin was used to block $GABA_A$ receptors in conjunction with ATPA. Picrotoxin has been shown to cause epileptic activity in the amygdala (Gean, P. W., & Shinnick-Gallagher, P. (1987)) and in our experiments, picrotoxin caused fEPSPs to become polysynaptic. This complicated the analysis of fEPSPs amplitudes, making them less reliable and stable due to polysynaptic noise.

There is also the additional issue that picrotoxin did not fully seem to block the effects of ATPA. While the reduction in amplitude was much larger when perfusing ATPA alone as compared to the perfusion of ATPA with picrotoxin, some kind of effect still seems to be taking place. A simple explanation could be that extended use of picrotoxin eventually causes the slice to deteriorate, due to spontaneous firing activity (Gean, P. W., & Shinnick-Gallagher, P. (1987)). This would cause the amplitude as well as the slope to drift down, independent of ATPA application. Extending the recordings by an additional 15 minutes or simply doing more experiments to increase the n-number would have shown if this is the case.

5.4. Concluding remarks

Kainate receptors remain an important aspect in understanding the developing brain and they also play a vital role in health and disease (Lerma, J., & Marques, J. M. (2013)). Physiological roles of kainate receptors at immature neuronal networks have been mainly studied in the hippocampus (Lauri et. al (2005) (2006), Vesikansa et al. (2012)). However, their contribution in the development of amygdalar networks remains under investigation. Here, we were able to show within a restricted developmental time window that activation of GluK1 subunit containing kainate receptors regulates synaptic transmission and plasticity in the basolateral amygdala indirectly, via recruitment of GABAergic inhibition. Interestingly, Arora et al. (2018) have recently shown that GluK4 subunit containing kainate receptors in the amygdala play a significant role in behavior and neuropsychiatric disease. The results highlight the fact that we must also take in to account different isoforms of kainate receptors in the amygdala, if we wish to understand their role in development and disease. Hence, it remains pertinent that we continue our investigations of these tantalizing receptors in the future.

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