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CHAPTER 5. BETA-ADRENERGIC SIGNALING TRIGGERS SYNAPTIC POTENTIATION AT CENTRAL AMYGDALA NEURONS (INDEPENDENTLY OF AMPA-RECEPTOR SUBUNIT COMPOSITION)

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Abstract

Norepinephrine has been implicated in emotional learning and arousal. Its direct effects on central amygdala (CeA) synapses have not been extensively studied. In this chapter we examine how beta-adrenergic signaling affects glutamatergic CeA synapses. We show that pharmacological activation of beta-adrenergic receptors induce synaptic potentiation in both SST⁺ and SST⁻ CeA neurons. To examine the role of different types of AMPA-receptors in this synaptic potentiation, we selectively knocked out either AMPA-receptor subunit GluA3 or GluA1. Interestingly, whereas the absence of GluA1 expression did not affect basal synaptic transmission, we observed that synaptic currents were increased in the absence of GluA3 expression, indicating that the presence of GluA3 dampens synaptic currents. However, beta-adrenergic stimulation was maintained in both GluA3- and GluA1-deficient neurons. Our data suggest that norepinephrine release in the CeA can potentiate glutamatergic synapses onto both SST⁺ and SST⁻ neurons independent of AMPA-receptor subunit composition.

5.1 Introduction

Norepinephrine (NE), one of the monoamines, has been implicated in emotional learning and arousal^{72,128}. The locus coeruleus (LC) is the major source of NE in the brain^{73,129}. NE is released from the LC into its projection sites, including the amygdala, and this release can be triggered by, for example, fearful events^{130,131}. NE can locally stimulate beta-adrenergic receptors, which activates adenylyl cyclases, resulting in increased cAMP levels¹³². Rising cAMP levels can in turn cause activation of glutamatergic receptors, causing synaptic alterations. Several glutamatergic receptors have been identified in the brain, including α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), N-methyl-D-aspartate (NMDA), kainate and metabotropic glutamate receptors. AMPA receptors (AMPA receptors) can be made up out of four different subunits, namely GluA1, GluA2, GluA3 and GluA4^{65,66,133}. The two most common types of AMPARs are thought to be heterodimers of GluA1/2s and GluA2/3s^{68,69}.

Since the central amygdala (CeA) has been implicated in emotional learning and arousal as well, it is of interest to examine the effects of NE on CeA synapses⁵. The CeA mainly consists of two inhibitory GABA-ergic cell populations: somatostatin (SST)-expressing and protein kinase C delta (Prkcd)-expressing neurons^{9,87}. In this chapter, we mimic the effects of NE on synapses of SST and Prkcd neurons, through the activation of beta-adrenergic signaling, and examine how this affects synaptic activity. Additionally, we examine the role of the different AMPAR subunits in beta-adrenergic signaling in the CeA.

5.2 CeA synaptic activity in the presence of beta-adrenergic stimulation

As a first step in determining how beta-adrenergic signaling affects CeA synapses, spontaneous excitatory post synaptic currents (sEPSCs) were recorded in the presence and absence of cAMP increasing drugs. Acute slices

were made from SST-IRES-cre;Ai14 animals in which SST neurons express red fluorescence; fluorescent neurons (SST⁺) as well as non-fluorescent neurons (SST⁻) could be identified. Isoproterenol, a β -adrenergic receptor agonist, and IBMX, a cAMP degradation blocker, were added to the perfusion system and mEPSCs were recorded in the presence and absence of these drugs.

5.2.1 Beta-adrenergic stimulation causes synaptic potentiation in SST⁺ and SST⁻ CeA neurons

No significant difference was observed in sEPSC frequency or amplitude in SST⁺ CeA neurons upon incubation with IBMX and isoproterenol (Figure 5.1A, B; unpaired t-test, all $p > 0.05$). The sEPSC frequency was increased in SST⁻ CeA neurons after incubation with IBMX and isoproterenol, with no effect on sEPSC amplitude (Figure 5.1C, D; unpaired t-test, $p = 0.008$, $p > 0.05$). This increase in spontaneous synaptic transmission in SST⁻ neurons could be a result of either a change in spontaneous network activity or an increase in synaptic strength. To examine this, we repeat our recordings in the presence of TTX to remove action potential-dependent vesicle release and measure events solely caused by spontaneous vesicle release (mEPSCs).

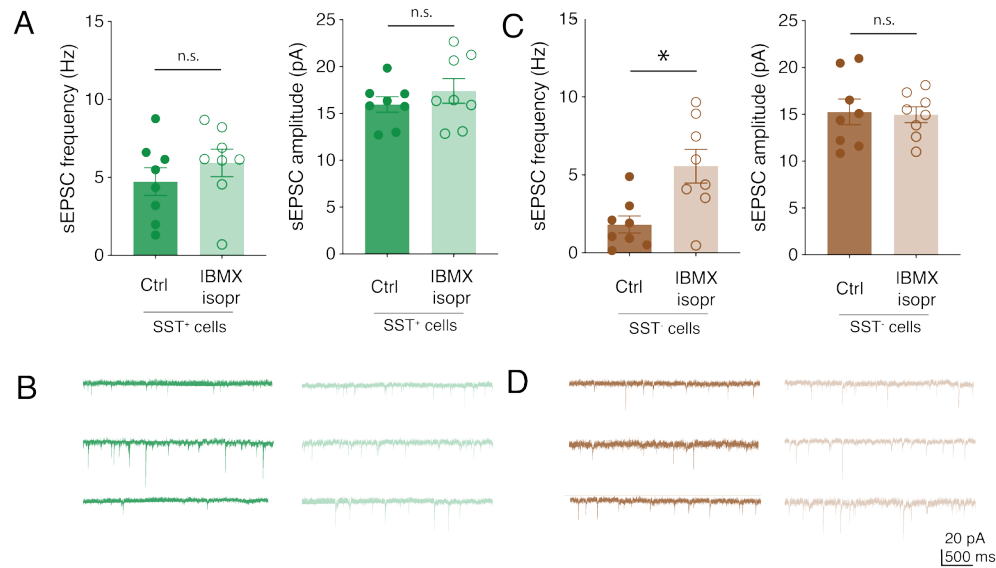


Figure 5.1 Beta-adrenergic stimulation increases spontaneous excitatory transmission in SST⁻ CeA neurons. (A) IBMX and isoproterenol did not affect sEPSC frequency or amplitude in SST⁺ CeA neurons (unpaired t-tests, all $p > 0.05$). (B) Representative sEPSC traces corresponding to data in panel (A). (C) IBMX and isoproterenol caused a significant increase in sEPSC frequency in SST⁻ CeA neurons (unpaired t-test, $p = 0.008$), while having no effect on sEPSC amplitude (unpaired t-test, $p > 0.05$). (D) Representative sEPSC traces corresponding to data in panel (C) Data represented as mean \pm s.e.m.

mEPSC frequency was increased in both SST⁺ and SST⁻ CeA neurons after incubation with IBMX and isoproterenol (Figure 5.2A, B, C, D; unpaired t-tests, $p = 0.0013$, $p = 0.0042$, respectively). IBMX and isoproterenol did not affect mEPSC amplitude (Figure 5.2A, B, C, D; unpaired t-tests, all $p > 0.05$). These data show that a rise in intracellular cAMP can increase synaptic strength in both SST⁺ and SST⁻ CeA neurons. Interestingly, we observed an increase in mEPSC frequency in SST⁺ neurons, while we see no effect in sEPSC frequency. It is possible that the action potential-dependent synapse and network activities overshadow the effect of IBMX and isoproterenol on synaptic strength in the sEPSC recordings. Interestingly, sEPSC frequency of SST⁺ neurons in control condition appears to be unusually high, suggesting that SST⁺ neurons might receive more action potential-dependent baseline input compared to SST⁻ neurons.

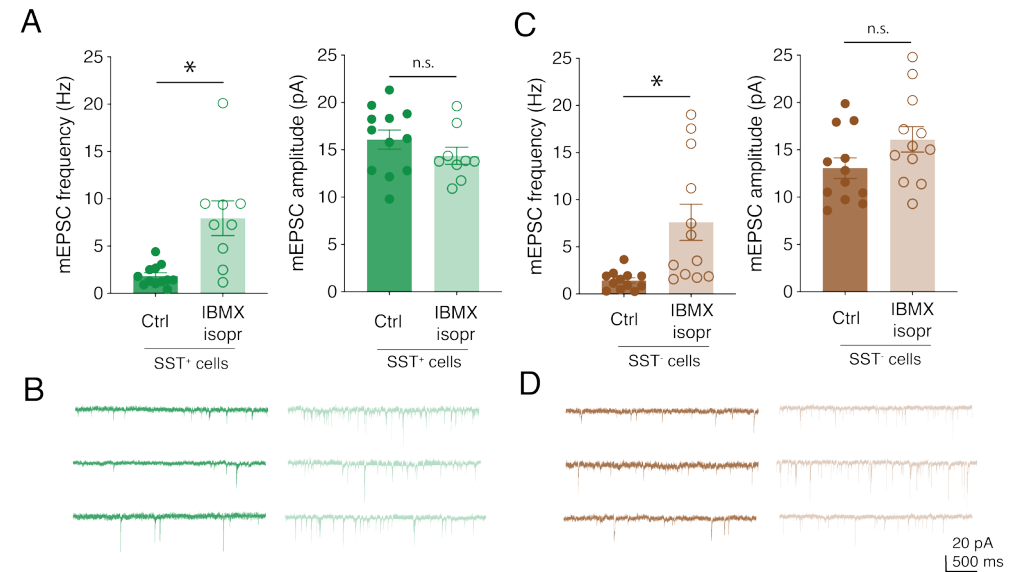


Figure 5.2 Beta-adrenergic stimulation induces synaptic potentiation in SST⁺ and SST⁻ CeA neurons. (A) IBMX and isoproterenol cause an increase in mEPSC frequency in SST⁺ CeA neurons (unpaired t-test, $p = 0.0013$), while no effect is seen on mEPSC amplitude (unpaired t-test, $p > 0.05$). (B) Representative mEPSC traces corresponding to data in panel (A). (C) IBMX and isoproterenol cause an increase in mEPSC frequency in SST⁻ CeA neurons (unpaired t-test, $p = 0.0042$), while no effect is observed on mEPSC amplitude (unpaired t-test, $p > 0.05$). (D) Representative mEPSC traces corresponding to data in panel (C). Data represented as mean \pm s.e.m.

5.2.2 CeA neurons express GluA1-4 subunits and removal of GluA3 causes an increase in basal transmission

Next, we wanted to know what kind of glutamatergic receptors (GluA1 or GluA3 containing AMPARs) were involved in the synaptic potentiation caused by a rise in intracellular cAMP. To examine this we used RNA-sequencing to first determine whether the different AMPAR subunits are expressed in CeA neurons. Additionally, we examine if the presence or absence of these subunits affect basal transmission.

Using RNAseq, we demonstrate that all four AMPAR subunits are expressed in both SST⁺ and SST⁻ CeA neurons (Figure 5.3A). However, the levels of GluA4 were lowest in both cell types, suggesting that the majority of AMPARs consist of GluA1/2 and GluA2/3. Since there do not seem to be major differences in the level of expression of the different subunits between SST⁺ and SST⁻ CeA neurons, and since no differences were found between SST⁺ and SST⁻ neurons in our mEPSC data, no distinction is made between the neuronal types in the remainder of this study.

Next, we examine if selective knockout of either the GluA3 or GluA1 subunit affects basal transmission. GluA3^{lox/lox} and GluA1^{lox/lox} animals were injected with a small amount of AAV9-GFP-IRES-cre virus, to knockout either GluA3 or GluA1 in neurons infected with the cre-virus, respectively. After 4 weeks of viral expression, acute slices were made and mEPSCs were recorded from WT (uninfected) and GluA3- or GluA1-deficient (GFP-expressing) neurons. Knockout of GluA3 caused an increase in mEPSC frequency, while knocking out GluA1 did not affect basal transmission (Figure 5.3B,C,D,E; unpaired t-tests, $p=0.0094$, $p>0.05$, respectively). These results suggest that the presence of GluA3 dampens synaptic transmission under basal conditions. Possibly, GluA3-containing AMPARs play a role in basal transmission in CeA neurons. Next, we assess if GluA3-containing AMPARs and GluA1-containing AMPARs are involved in the synaptic potentiation caused by a rise in intracellular cAMP.

5.2.3 CeA neurons from GluA3-knockout animals show synaptic potentiation upon beta-adrenergic stimulation

To examine the possibility that the synaptic potentiation we observe is based on GluA3-containing AMPAR plasticity, GluA3^{lox/lox} animals were crossed with ACTB-cre animals to generate animals in which GluA3 is depleted in all beta-actin-expressing cells¹³⁴. Acute slices were made and CeA neurons were recorded in the presence and absence of IBMX and isoproterenol.

To assess if the synaptic potentiation induced by beta-adrenergic stimulation in CeA neurons is dependent on GluA3-containing AMPARs, mEPSCs from CeA neurons in GluA3-deficient animals are recorded. Application of IBMX and isoproterenol could robustly increase mEPSC frequency in GluA3-deficient animals, while not affecting mEPSC amplitude (Figure 5.4A,B; unpaired t-tests, $p=0.0332$, $p>0.05$, respectively). These results are comparable to the effects we see from IBMX and isoproterenol in wildtype animals, suggesting that the cAMP-mediated increase in mEPSCs from CeA neurons is not dependent on the presence of GluA3-containing AMPARs. However, there is always a possibility with chronic knockout animals, that compensatory mechanisms can alleviate any effects from the knockout. To control for this, we next recorded from animals that have both WT CeA neurons and GluA3-deficient CeA neurons.

5.2.4 Beta-adrenergic driven synaptic potentiation of CeA neurons is not mediated by GluA3-containing AMPARs

GluA3^{lox/lox} animals were injected with a small amount of AAV9-GFP-IRES-cre virus, to knockout GluA3-containing AMPARs in neurons infected with the cre-virus. After 4 weeks of viral expression, acute slices were made and mEPSCs were recorded from WT (uninfected) and GluA3-deficient (infected) neurons. IBMX and isoproterenol could increase mEPSC frequency in both WT and GluA3-deficient neurons (Figure 5.5A,B,C,D; unpaired t-tests, $p=0.0468$, $p=0.0022$, respectively). Since mEPSCs are recorded from both WT neurons and GluA3-deficient neurons in the same brain slice, it is possible to compare the magnitude of the cAMP-induced synaptic potentiation. No differences were found in fold changes of mEPSC frequency and mEPSC amplitude between WT and GluA3-deficient neurons (Figure 5.5E; unpaired t-tests, all $p>0.05$). These results suggest that cAMP induced synaptic potentiation in CeA neurons is not dependent on GluA3-containing AMPARs. Alternatively, it is possible that cAMP-induced synaptic potentiation in the CeA is dependent on GluA1-containing AMPARs.

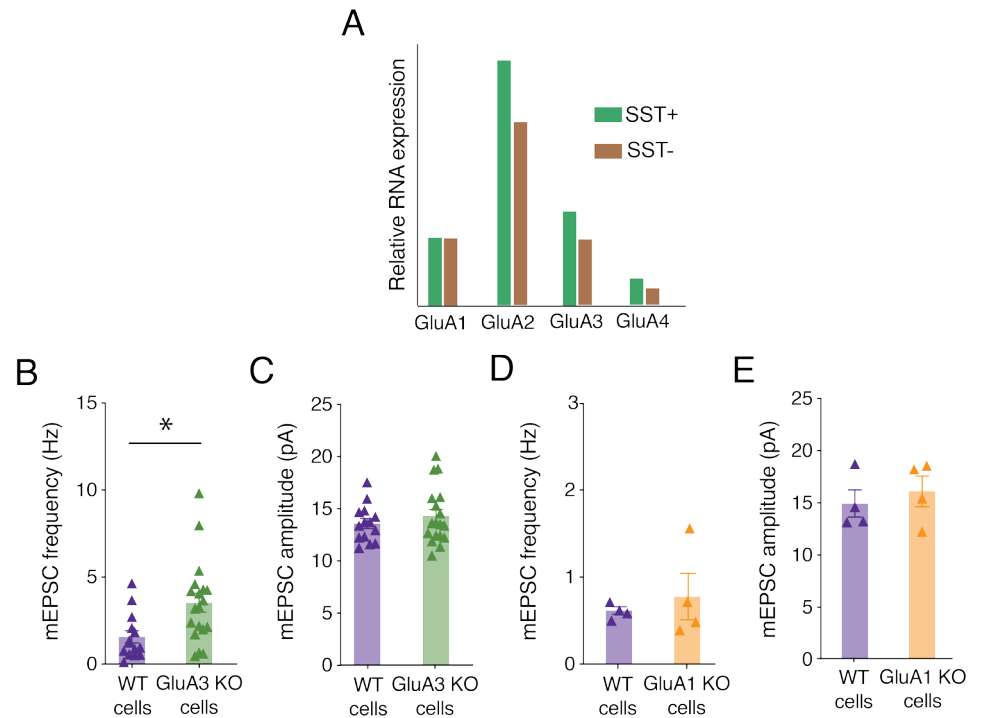


Figure 5.3 Subunits GluA1-4 are expressed in SST⁺ and SST⁻ CeA neurons and removal of GluA3 increases basal synaptic transmission. (A) Relative RNA expression demonstrates the presence of GluA1, GluA2, GluA3 and GluA4 subunits in both SST⁺ and SST⁻ CeA neurons. (B) mEPSC frequency in GluA3 KO cells is significantly increased compared to WT cells (unpaired t-test, $p=0.0094$). (C) No differences are found between mEPSC amplitude of WT and GluA3 KO cells (unpaired t-test, $p>0.05$). (D) No differences are found in mEPSC frequency between GluA1 KO cells and WT cells (unpaired t-test, $p>0.05$). (E) No differences are found between mEPSC amplitude of WT and GluA1 KO cells (unpaired t-test, $p>0.05$). Data represented as mean \pm s.e.m.

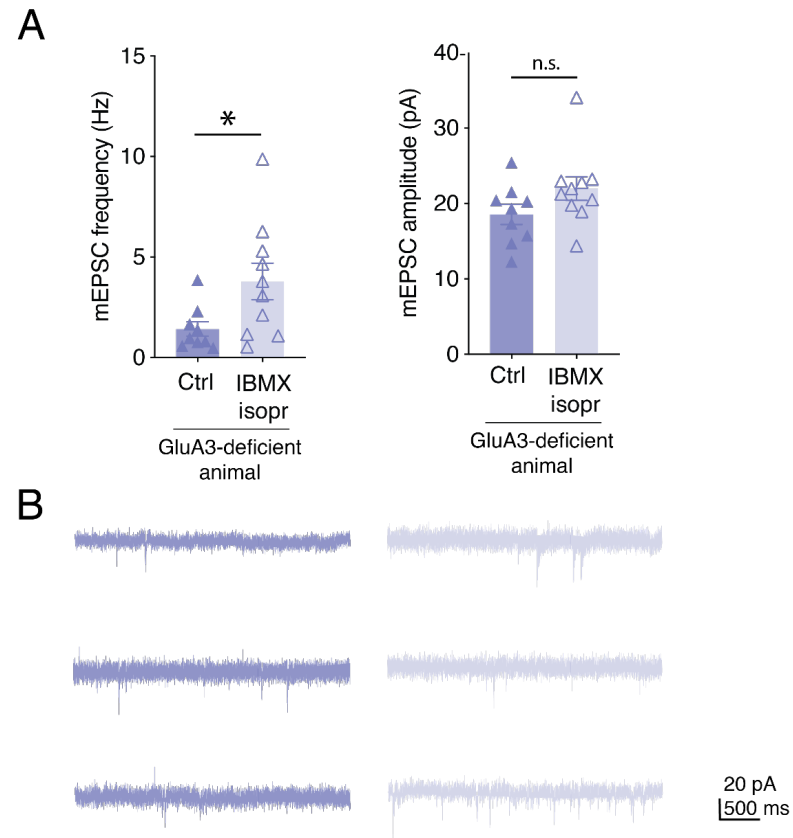


Figure 5.4 IBMX and isoproterenol-induced synaptic potentiation of CeA neurons in GluA3-deficient animals. (A) IBMX and isoproterenol application increases mEPSC frequency in CeA neurons from GluA3-KO animals (unpaired t-test, $p=0.0332$), while not affecting mEPSC amplitude (unpaired t-test, $p>0.05$). (B) Representative mEPSC traces corresponding to data in panel (A). Data represented as mean \pm s.e.m.

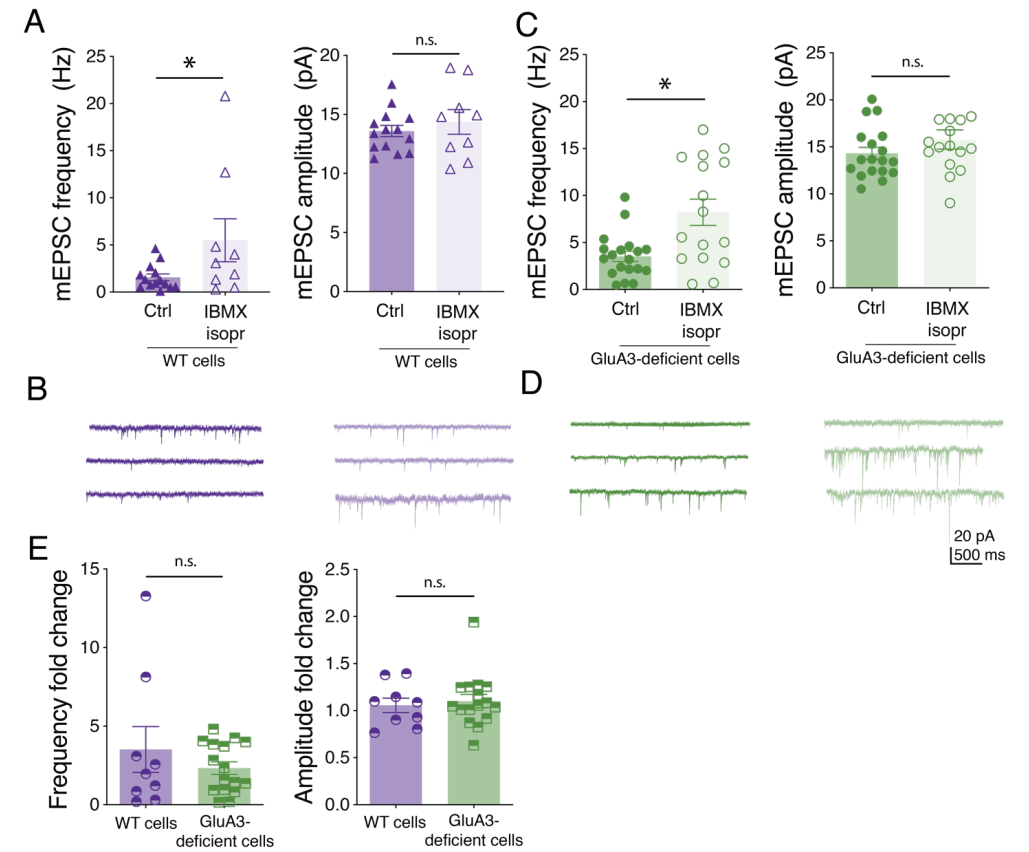


Figure 5.5 IBMX and isoproterenol-induced synaptic potentiation of CeA neurons is not dependent on GluA3-containing AMPARs. (A) IBMX and isoproterenol application increases mEPSC frequency in CeA neurons with intact GluA3-containing AMPARs (unpaired t-test, $p=0.0468$), while not affecting mEPSC amplitude (unpaired t-test, $p>0.05$). (B) Representative mEPSC traces corresponding to data in panel (A). (C) IBMX and isoproterenol applications increases mEPSC frequency in GluA3-deficient neurons (unpaired t-test, $p=0.0022$). (D) Representative mEPSC traces corresponding to data in panel (C). (E) When comparing the frequency and amplitude fold change after IBMX and isoproterenol application, no differences are found between WT and GluA3-deficient neurons (unpaired t-tests, all $p>0.05$). Data represented as mean \pm s.e.m.

5.2.5 Dependence of GluA1 on beta-adrenergic driven synaptic potentiation of CeA neurons (preliminary)

To assess the role of GluA1-containing AMPARs in CeA neurons during high intracellular cAMP levels, GluA1^{lox/lox} animals were injected with a small amount of AAV9-GFP-IRES-cre, to knockout GluA1-containing AMPARs in neurons infected with the cre-virus. After 4 weeks of viral expression, acute slices were made and mEPSCs were recorded from GluA1-deficient (infected) neurons. IBMX and isoproterenol administration did not significantly affect mEPSC frequency or amplitude in GluA1-deficient cells (Figure 5.6AB; unpaired t-tests, all $p > 0.05$). However, it seems there might be a trend towards an increase in mEPSC frequency in these cells. These results are obviously preliminary, so more animals need to be added. Nevertheless, if there is indeed no difference between Ctrl and IBMX+isoproterenol treated neurons, this would point towards a role of GluA1-containing AMPARs in cAMP-induced synaptic potentiation in CeA neurons. However, if the trend towards an increase in mEPSC frequency after IBMX and isoproterenol administration continues, it is likely that GluA1-containing AMPARs do not play a role in cAMP-induced synaptic potentiation, which seems most reasonable. It is crucial to add more recordings before a definitive conclusion can be drawn.

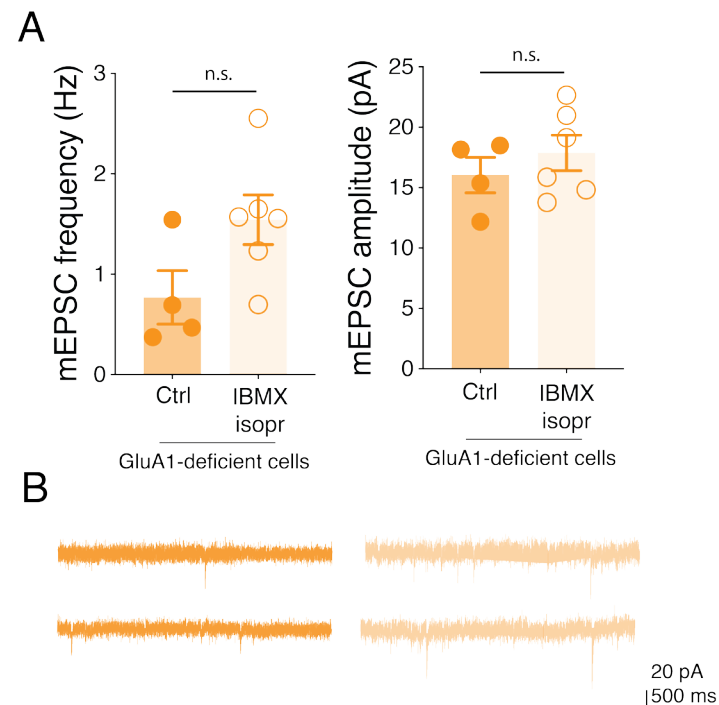


Figure 5.6 IBMX and isoproterenol-induced synaptic potentiation of CeA neurons might be partly dependent on GluA1-containing AMPARs. (A) IBMX and isoproterenol application has no effect on mEPSC frequency or mEPSC amplitude in GluA1-deficient CeA neurons (unpaired t-test, all $p > 0.05$). (B) Representative mEPSC traces corresponding to data in panel (A). Data represented as mean \pm s.e.m.

5.3 Discussion

In this chapter, we tried to examine how beta-adrenergic signaling could affect CeA synapses. Firstly, we showed that the pharmacological stimulation of beta-adrenergic receptors could induce synaptic potentiation in both SST⁺ and SST⁻ CeA neurons, as observed through an increase in mEPSC frequency. Interestingly, a previous study showed that NE could inhibit synapses in the CeA through a presynaptic mechanism that decreases the number of active release sites, but they did not observe an effect from NE on miniature EPSCs¹³⁵. It is possible that we are measuring different types of synapses. A change in mEPSC frequency, as we have seen in our results, has been found to either reflect a change in presynaptic vesicle release probability, or a change in the number of active synapses. Based on these experiments, no definitive conclusion can be drawn about the mechanism underlying the increase in mEPSC frequency. However, similar results have been obtained by Renner *et al.*¹³⁶ who have shown the ability of CA1 hippocampal neurons to exhibit synaptic potentiation upon an increase in cAMP, as demonstrated by an increase in mEPSC frequency. They concluded that the change in mEPSC frequency was caused by an increase in the number of active synapses, since they did not observe an altered paired pulse ratio or a change in quantal content of evoked glutamate release. Based on these results from Renner *et al.*, it would be possible that the cAMP dependent synaptic potentiation seen in CeA neurons in the current study, is also caused by an increase in the number of active synapses that are detectable in mEPSC recordings. However, additional experiments on paired pulse ratio and quantal content should be carried out to confirm this. In addition, Renner *et al.*¹³⁶ demonstrated that the cAMP dependent synaptic potentiation in hippocampal neurons was dependent on GluA3-containing AMPARs, as the effect was absent in slices from GluA3-deficient mice.

Since we showed that beta-adrenergic signaling caused a robust synaptic potentiation, we next wanted to examine how different AMPAR subunits were involved in this effect. First, we showed that AMPAR subunits GluA1-4 were all expressed in both SST⁺ and SST⁻ CeA neurons, and assessed how the presence of the subunits affects basal transmission. Surprisingly, we demonstrate that removal of GluA3-containing AMPARs causes an increase in synaptic activity, suggesting that the presence of GluA3-containing dampens synaptic transmission under basal conditions. GluA3-containing AMPARs were shown to have a reduced channel conductance compared with GluA1-containing ones¹³⁶. Possibly the replacement of GluA1/2s by low-conductive GluA2/3s may lead to reduced basal synaptic transmission at CeA synapses.

Next, we sought to examine if the synaptic potentiation we observe is also dependent on GluA3-containing AMPARs. However, when we repeated experiments in global GluA3 knockouts and cellular GluA3 knockouts, we saw that synaptic potentiation was still possible, leading us to conclude that our cAMP induced synaptic potentiation is not dependent on GluA3-containing AMPARs. Additionally, we repeated the experiment in GluA1 global knockouts and from our preliminary data we saw that there was a trend towards the cAMP dependent potentiation. It is of importance to increase the number of recordings to confirm this result.

In this chapter, we have demonstrated that beta-adrenergic signaling causes synaptic potentiation at glutamatergic CeA synapses. Our result show that this effect is not caused by specific postsynaptic AMPARs, so it is possible that we are observing a presynaptic effect. However, we cannot exclude a postsynaptic effect that is independent of AMPAR subunit composition.