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The Role of Calcium-Permeable Kainate and Ampa Receptors in The Leading Reaction of Gabaergic Neurons to Excitation

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Abstract

While all neurons can intrinsically generate action potentials upon depolarization, those in the central nervous system do not fire constantly in response to numerous excitatory signals. This stability is attributed to control by an inhibitory GABAergic system. For this system to effectively prevent hyperexcitability, GABAergic neurons must generate inhibitory signals before glutamatergic excitation occurs, implying a faster response capability.This study demonstrates that in mature hippocampal cultures (14 DIV) and in brain slices from two-month-old rats, neurons do not respond simultaneously to general depolarization (induced by KCl, NH4Cl, or cAMP) or to glutamate receptor agonists (domoic acid, glutamate). Consistent with the above hypothesis, most GABAergic neurons fire before glutamatergic neurons. The delay in glutamatergic response is inversely proportional to stimulus intensity and can last dozens seconds. Crucially, this delay is abolished by GABA(A) receptor inhibitors, indicating a mechanism of preliminary GABA release. We show that early-responding GABAergic neurons express low-threshold, calcium-permeable kainate and AMPA receptors (CP-KARs and CP-AMPARs), which they use to generate a rapid response. However, when domoic acid application was repeated in the presence of the AMPA receptor inhibitor NBQX, the response delay was significantly increased. This result confirms that low-threshold CP-KARs localized on the presynaptic terminals of GABAergic neurons are responsible for the delay in glutamatergic neuron excitation. In hippocampal slices from two-month-old rats, overall depolarization with 50 mM KCl elicited distinct calcium responses in two neuronal populations. The majority of neurons (presumably glutamatergic) showed fluctuating Ca^{2+} signal, while a smaller group (presumably GABAergic) exhibited a steady, advancing $[Ca^{2+}]i$ increase. The subsequent application of domoic acid reinforced this distinction. Neurons that displayed an early, advancing Ca^{2+} response to KCl also responded to domoic acid with a similar advancing increase. In contrast, neurons that responded to KCl with delayed fluctuations also showed fluctuating responses to domoic acid, but with an even longer delay (80 s). Therefore, these experiments identify a subgroup of hippocampal neurons—both in slices and in culture—that respond with an early $[Ca^{2+}]i$ signal to both depolarization and glutamate receptor agonists (domoic acid, glutamate). Consistent with findings from cell cultures, we conclude that these early-responding neurons are GABAergic. Their early GABA release likely explains the delayed response observed in the glutamatergic neuron.

Keywords: early signal of GABAergic neurons; calcium-permeable CP-KA и CP-AMPA receptors; low-threshold spiking (LTS) interneurons; Ca^{2+} imaging

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1. Introduction

Previous research has established that calcium-permeable kainate and AMPA receptors (CP-KARs and CP-AMPARs) are predominantly expressed in hippocampal GABAergic neurons of adult rats [1]. These receptors define two major subgroups of neurons, which together constitute over 55% of the GABAergic population [1,2]. One group expresses CP-KARs, and the other expresses CP-AMPARs [1–4]. CP-AMPAR-expressing GABAergic neurons innervate those expressing CP-KARs, and to a lesser extent, the reverse is also true [1,2,5]. Functionally, these receptors act as low-threshold ion channels, enabling neurons to be excited by weak depolarizations or low concentrations of agonists like domoic acid (DoA) [1,3]. For instance, GABAergic neurons expressing these receptors generate a sodium current at approximately -55 mV, a potential requiring only minimal depolarization. In contrast, glutamatergic neurons typically require a stronger depolarization to reach their excitation threshold of around -42 mV [3]. Agonists like ATPA (targeting GluR5 subunits of CP-KARs) or SYM2081 selectively increase intracellular calcium in CP-KAR-expressing neurons, induce GABA release, and consequently suppress the activity of downstream neurons [5–8]. These properties have even been leveraged to develop methods for visualizing these specific neurons in culture [3,9]. Notably, during DoA exposure, these GABAergic neurons exhibit a unique response: they are not fully excited to fire action potentials. Instead, calcium influx through CP-KARs reaches a subthreshold level that is sufficient to trigger GABA release directly.

This functional profile aligns with that of low-threshold spiking (LTS) interneurons, which are known to provide advanced inhibition and are excited by small shifts in membrane potential [10,11]. While classic feedforward inhibition in LTS interneurons is often attributed to T-type calcium channels (also activated at -55 mV), the receptor-mediated mechanism described here provides an alternative pathway. It is important to note a discrepancy in the population size: while LTS interneurons are estimated to comprise 4.5-15% of hippocampal GABAergic neurons [25], our data indicate that over 55% express CP-KARs or CP-AMPARs in culture [1]. This suggests that these low-threshold receptor phenotypes may be more widespread among GABA neurons than the defined LTS interneuron subtype.

This study demonstrates that GABAergic neurons expressing CP-KARs and CP-AMPARs respond earlier than glutamatergic neurons, both to depolarization and to glutamate receptor agonists in slices of the hippocampus from adult two-month-old animals. Thus, the mechanisms of the preliminary reaction of GABAergic neurons and the delay in the excitation of other neurons are realized in the brains of adult animals.

2. Results

2.1. GABAergic Neurons Expressing CP-KARs and CP-AMPARs Receptors

We have previously established simple methods to visualize distinct populations of GABAergic neurons [9,12]. To identify neurons expressing CP-KARs we detect a rise in $[Ca^{2+}]_i$ following application of the selective agonist ATPA. To identify neurons expressing CP-AMPARs we apply domoic acid and then block the subsequent Ca^{2+} signal with the selective antagonist NASPM.

Figure 1 illustrates a representative experiment tracking changes in $[Ca^{2+}]_i$ in two GABAergic neuron subtypes: those expressing CP-KARs (black curves) and those expressing CP-AMPARs (red curves). Application of domoic acid (DoA), an agonist for both receptors, increased $[Ca^{2+}]_i$ in both subtypes. The response was faster in ATPA-sensitive neurons (CP-KARs) and slower in NASPM-sensitive neurons (CP-AMPARs). The subtype-specificity of the responses was confirmed by applying NASPM, which inhibited the DoA-induced Ca^{2+} signal in CP-AMPAR-expressing neurons, and ATPA, which selectively induced a $[Ca^{2+}]_i$ rise in CP-KAR-expressing neurons. Immunostaining verified that both neuronal subtypes are GABAergic [1]. This functional distinction complemented with the known subcellular localization of these receptors: CP-KARs are primarily presynaptic [13–15], while CP-AMPARs are postsynaptic [13,16–18].

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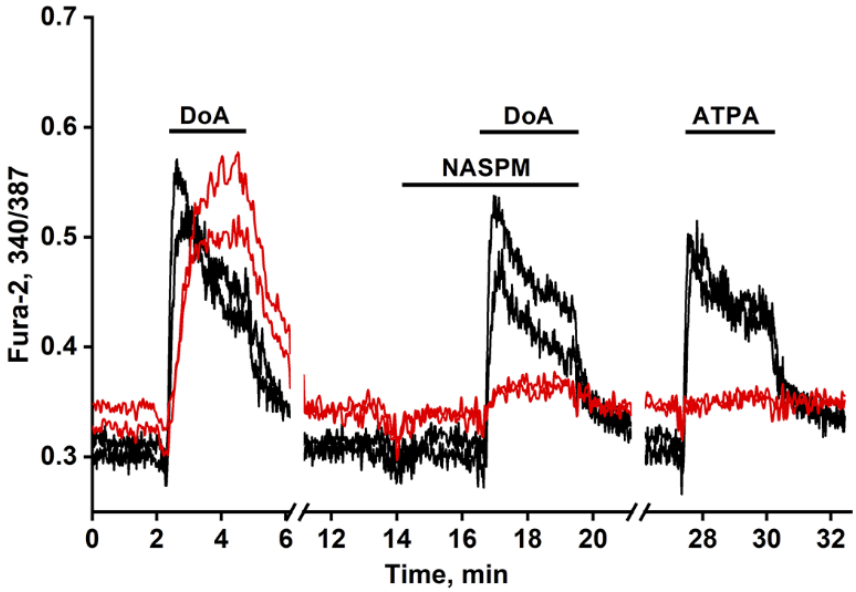

Figure 1. Dynamic of $[Ca^{2+}]_i$ changes in two GABAergic neurons expressing CP-AMPARs (red curves) and in two expressing CP-KARs (black curves). Both subtypes respond to DoA (350 nM). In one of the subtypes responding more slowly to DoA the response is inhibited by NASPM (10 μM), while the other, responding more quickly, is activated by ATPA (350 nM).

2.2. The Advancing Response of Neurons to DoA is Eliminated by GABA(A) Receptor Inhibitors

Sequential pharmacological manipulation in an oscillating neuronal culture (14 DIV) revealed distinct $[Ca^{2+}]_i$ dynamics in response to hyperexcitation (Figure 2). Application of DoA, an agonist for both KA and AMPA receptors, induced a biphasic response: a rapid $[Ca^{2+}]_i$ increase in a small subpopulation of neurons (14% ± 5%; red curves) and a delayed response in the majority.

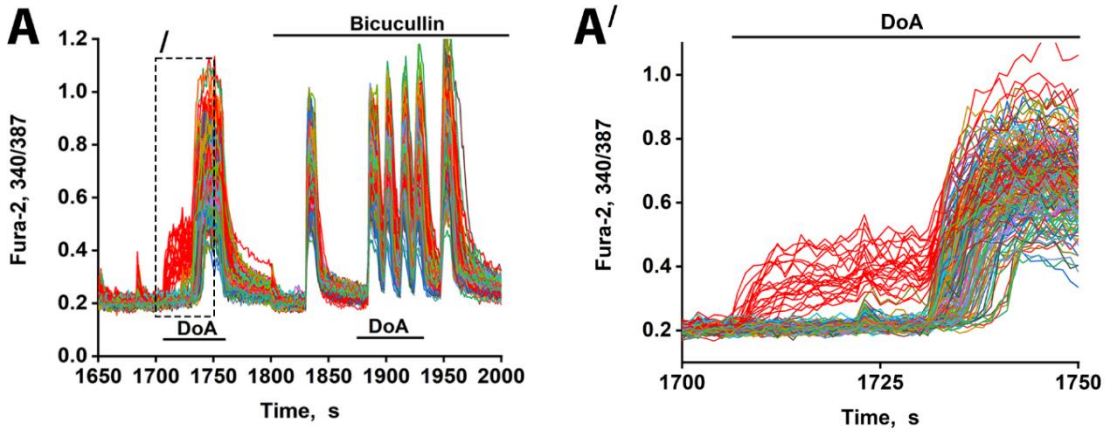

Figure 2. Change in $[Ca^{2+}]_i$ in neurons in response to 100 nM DoA in control and in the presence of 10 μM bicuculline. (**A**) - The delay in the response of glutamatergic neurons to DoA is removed by bicuculline. From 23 to 33 out of 200 cells responded with an early signal (red curves). (**A′) -** The left part of Figure 2A (indicated by dashed lines). The delay is 30 ± 5 s. GABAergic neurons desynchronize other neurons.

Consistent with prior studies [1–3], the neurons exhibiting rapid responses are GABAergic and express calcium-permeable KARs and AMPARs. Inhibition of GABA(A) receptors with bicuculline abolished the delayed excitation, confirming GABA's involvement. Bicuculline also increased the amplitude of calcium spikes, an effect attributable to depolarization resulting from postsynaptic Cl^-

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channel closure. The extended timeline (Figure 2A') further shows that the delayed responses were not synchronous, likely reflecting heterogeneity in the Cl^- ion gradient across neurons which influence the membrane potential shift upon GABA(A) channel opening [19]. Therefore, beyond simply delaying excitation, GABAergic activity serves to desynchronize the network's response to hyperexcitatory stimuli.

2.3. The Excitation Delay Depends on the Concentration of the Agonist and is Determined by CP-KARs

As we have previously demonstrated, DoA selectively activates kainate receptors (KARs) when AMPARs are blocked by NBQX [1]. Under these conditions, a low concentration of DoA (100 nM) triggered an immediate response in GABAergic neurons expressing calcium-permeable KARs (CP-KARs), while inducing a significant delay (~80 s) in the excitation of glutamatergic neurons (Figure 3A). This delay was concentration-dependent, as increasing DoA to 500 nM substantially reduced the latency to excitation, consistent with an increased depolarizing drive (Figure 3C). In the presence of bicuculline, the delay is still evident at the low agonist concentration (Figure 3B), but it was completely absent at high DoA concentration (Figure 3D).

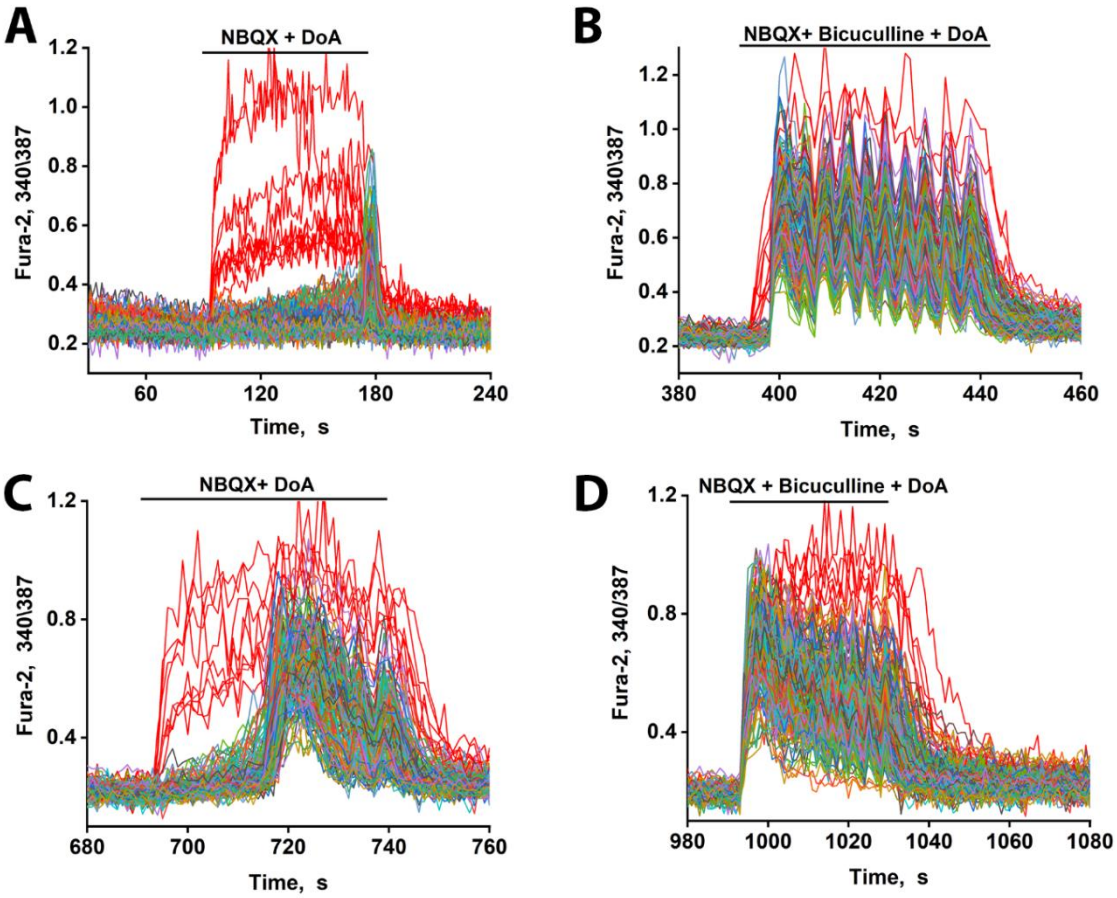

Figure 3. Change in $[Ca^{2+}]_i$ in neurons in response to DoA. (**A**) - Effect of DoA (100 nM) in the presence of the AMPA receptor inhibitor, 2 μM NBQX. An early response of GABAergic neurons expressing CP-KARs (red curves) is shown. Glutamatergic neurons (gray-blue-green curves) respond with a delay of 80 s. (**B) -** Change in $[Ca^{2+}]_i$ in neurons in response to 100 nM DoA in the presence of 10 μM bicuculline. (**C) -** When the DoA concentration is increased to 500 nM, the delay in the response of glutamatergic neurons (gray-blue-green curves) decreases from 80 s to 20 s. (**D) -** Bicuculline (10 μM) completely removes the delay in the case of a high concentration of the DoA (500 nM).

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These results indicate that while both subtypes of GABAergic neurons respond early to DoA, but the inhibitory delay in glutamatergic neurons is primarily mediated by CP-KARs on the corresponding GABAergic neurons.

2.4. Early Response of DoA-Sensitive GABAergic Neurons to Glutamate

Unlike NMDA receptors, which require depolarization to relieve Mg^{2+} block, calcium-permeable AMPARs and KARs (CP-AMPARs/CP-KARs) permit immediate Ca^{2+} influx without prior depolarization. We therefore hypothesized that neurons expressing these receptors would respond to glutamate more rapidly, particularly at low concentrations.

In mature cultures (14 DIV), glutamate indeed elicited a biphasic Ca^{2+} response: an early response in a minority of neurons and a delayed response in all other neurons (Figure 4A). The early-responding neurons were the same GABAergic cells that responded rapidly to DoA (red curves, Figure 4B) [2,3].

Additionally, glutamate triggered a rapid response in a small subset of other neurons (6-8 out of 200; green curves, Figure 4A, C). These neurons are likely not GABAergic, as they did not respond early to DoA. Instead, they appear to be innervated by the DoA-sensitive GABAergic neurons, as they responded to DoA with a prolonged delay (~3 s longer than average; Figure 4D). This extended delay may indicate a significant Cl^- ion gradient in these cells. Alternatively, they might possess a higher affinity for glutamate. Unlike the DoA-sensitive GABAergic neurons, these glutamatergic neurons exhibited asynchronous oscillatory activity upon glutamate application —a pattern characteristic of mild depolarization.

These results demonstrate that GABAergic neurons expressing CP-KARs and CP-AMPARs provide the earliest response to glutamate. This rapid excitation is consistent with their previously demonstrated rapid response kinetics [1,3]. Through their early activation, these neurons delay the firing of their target's neurons, thereby desynchronizing network activity.

2.5. Early Response of DoA-Sensitive GABAergic Neurons to Depolarization

GABAergic neurons expressing calcium-permeable AMPARs and KARs (CP-AMPARs/CP-KARs) are poised for rapid activation. These receptors are low-threshold [1,3], and

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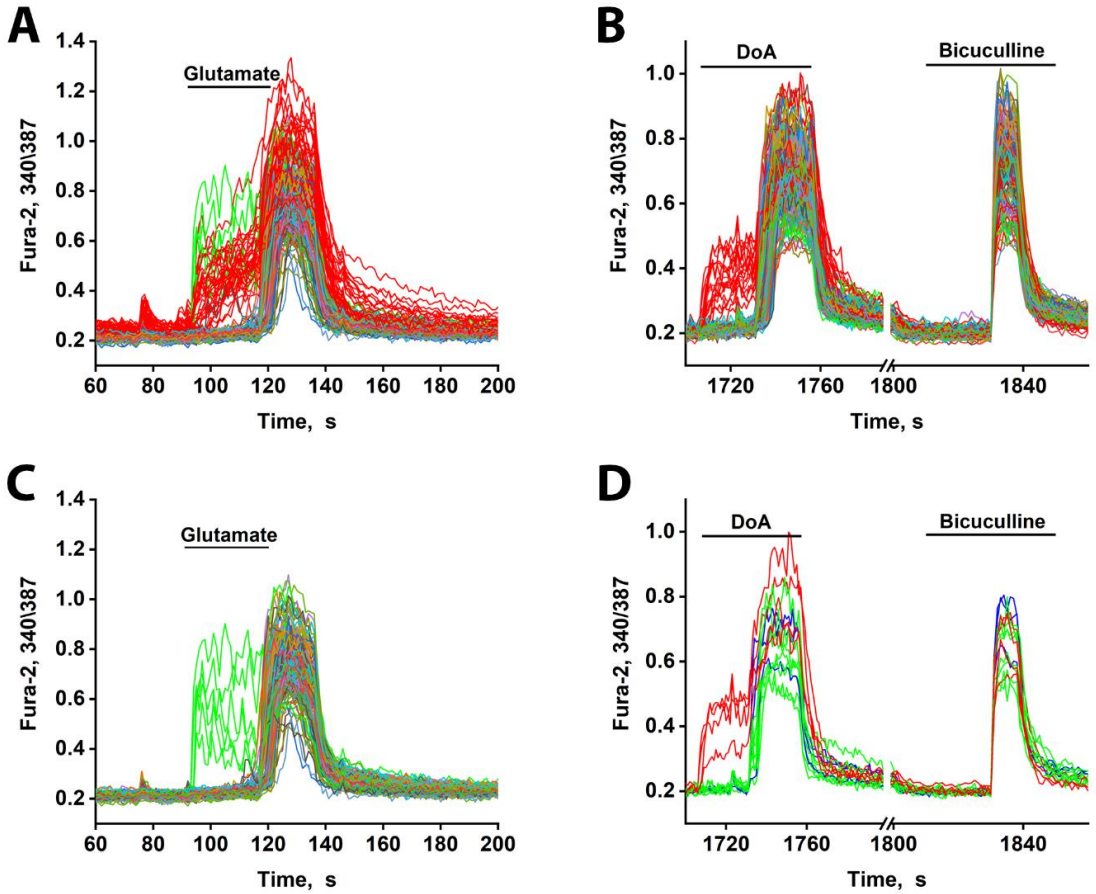

Figure 4. $[Ca^{2+}]_i$ changes in neurons in response to 10 μM glutamate and DoA. (**A**) - The minor subtypes of neurons exhibits an early reaction to glutamate (red and green curves). All DoA-sensitive neurons participate in the early response to glutamate (red curves). The duration of the delay is about 30 s. (**B**) - An early response of GABAergic neurons (red curves) to 100 nM DoA. (**4C**) - 10 μM Glutamate induces a rapid oscillatory response in 3–4% of neurons, (green curves), which are not part of the DoA-sensitive neuron subtype. (**D**) - The neurons marked in green in Figures 4A and 4C respond to DoA with a significant delay. This delay is eliminated by bicuculline. However, during strong depolarization caused by the bicuculline, these neurons oscillate synchronously with the others neurons. Data are from a sample of 200 neurons in the field of view.

the somata of these neurons receive minimal inhibitory input [9]. Consequently, they can depolarize earlier than other neurons in response to a global stimulus, leading to GABA release [7]. We tested this using a moderate depolarization (10-12 mV) induced by 2-8 mM NH_4Cl, which alters membrane potential by passing through potassium channels [1,20]. In one experiment (Figure 5A), 8 mM NH_4Cl triggered an immediate $[Ca^{2+}]_i$ increase in two GABAergic neuron subtypes (red and blue curves) but a delayed response in a glutamatergic neuron (green curve). The glutamatergic population was excited after a 30-40 s delay, followed by synchronous oscillations.

Pharmacological profiling confirmed the identity of the early responders: pretreatment with the CP-KAR (GluK1-containing) agonist ATPA identified one GABAergic subtype (red curve), while the CP-AMPAR antagonist NASPM inhibited the other (blue curve, data not shown). This confirms that both CP-AMPARs and CP-KARs are low-threshold and activate before other calcium channels upon moderate depolarization. The resulting Ca^{2+} influx in these neurons triggers GABA release [5–7], which delays the excitation of their targets. As with DoA and glutamate, this delay was abolished by bicuculline (data not shown). The sharp onset of the delayed signal suggests a cooperative mechanism involving positive feedback.

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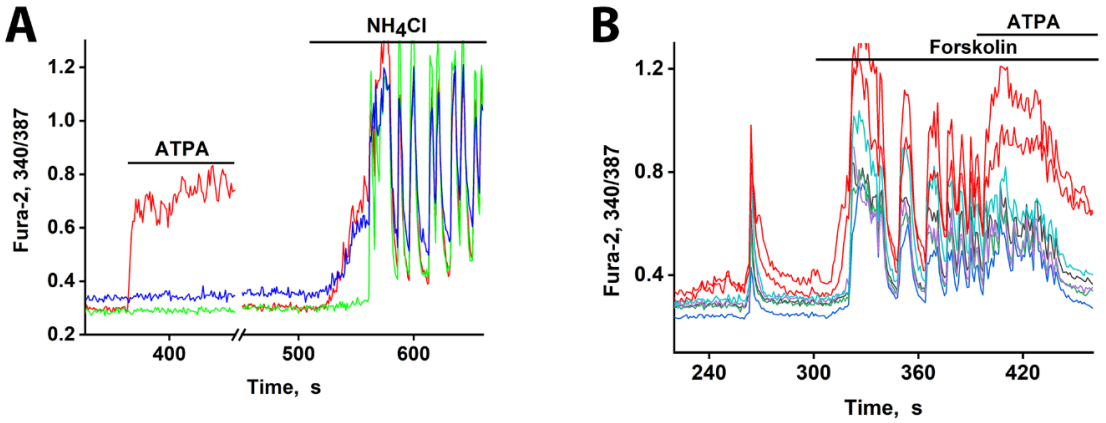

Figure 5. $[Ca^{2+}]_i$ changes in representative neurons in response to depolarization. (A) – The response of neurons from three subgroups to depolarization induced by NH_4Cl (8mM). ATPA-sensitive GABAergic neuron (red curve). GABAergic neuron expressing CP-AMPARs (blue curve). Glutamatergic neuron reacts after a delay of 30 s (green curve). Synchronization of oscillations occurs after 20 s of general excitation. 240 neurons in the field of view. (**B**) **-** An early response to forskolin (70 μM) is observed in neurons that selectively react to ATPA (200 nM) with $[Ca^{2+}]_i$ increase (red curves).

In conclusion, the early response to NH_4Cl-induced depolarization is mediated by DoA-sensitive GABAergic neurons expressing CP-AMPARs and CP-KARs. However, only the GABAergic neurons expressing CP-KARs are responsible for imposing the excitation delay on glutamatergic neurons, as they provide the direct inhibitory innervation (Figure 3).

2.6. GABAergic Neurons Exhibit an Early Response to cAMP Elevation

cAMP typically promotes neuronal excitation by enhancing Na^+ and Ca^{2+} currents while inhibiting K^+ currents, leading to depolarization and increased network oscillations [21–23]. However, this excitation has a dual effect: when it occurs in GABAergic interneurons, it enhances inhibitory tone. The increased firing in these interneurons and relatively slow reuptake of GABA elevates their basal $[Ca^{2+}]i$, facilitating GABA release and resulting in the inhibition of downstream target neurons [6].

Our data reveal that this cAMP-mediated excitation is not simultaneous across all neurons. Figure 5B demonstrates that interneurons expressing calcium-permeable kainate receptors, identified by their response to ATPA, are the first to respond to forskolin-induced cAMP elevation. Note that the short response delay observed in this experiment, is likely attributable to a high baseline level of excitation, as indicated by the amplitude of spontaneous synchronous, as well as elevated cAMP. Consequently, GABAergic neurons expressing CP-KARs and CP-AMPARs are uniquely sensitive, responding rapidly to both direct glutamatergic agonists and to moderate depolarizing signals like cAMP.

2.7. The Early GABAergic Neuron Response to Excitation in Hippocampal Slices

To determine if the early response of GABAergic neurons to excitation also occurs ex vivo, we used hippocampal slices from the CA1 area of two-month-old rats. As shown in Figure 6A, general depolarization with 50 mM KCl elicited distinct response patterns: the majority of neurons displayed oscillatory Ca^{2+} transients (presumed glutamatergic; blue-green curves), whereas a small subset exhibited a rapid, non-oscillatory $[Ca^{2+}]_i$ rise (presumed GABAergic; red curves). Response delays among glutamatergic neurons were under 10 s. All responses were reversible upon washout. The oscillatory activity, occurring at approximately 0.2 Hz, is notable as it matches the frequency of epileptiform activity observed in neuronal cultures.

To pharmacologically validate the identity of the early-responding cells, we applied DoA, an activator of kainate and AMPA receptors (Fig. 6B). Consistent with the KCl response, a subset of

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neurons (three in Fig. 6C) displayed an immediate $[Ca^{2+}]_i$ increase, while the majority responded with delayed oscillations. Subsequent DoA application triggered an early response in two of the three neurons that responded early to KCl (Figs. 6B, D), confirming their sensitivity to this glutamatergic agonist and supporting their classification as GABAergic interneurons. The population of neurons that responded to KCl with delayed oscillations also responded to DoA with oscillations, but after a significantly longer delay (80 s versus 30 s). The same neurons are color-coded across panels. One neuron that responded early to KCl did not respond early to DoA, indicating it was likely glutamatergic; a similar phenomenon also observed in cultured neurons exposed to glutamate and NH_4Cl (Fig. 4).

These results demonstrate that a subset of neurons in hippocampal slices from adult rats, like those in culture, exhibits an early Ca^{2+} response to both depolarization and the kainate/AMPA receptor agonist DoA. Based on evidence from cell culture, these rapidly responding cells are GABAergic and express calcium-permeable KARs and AMPARs (CP-KARs/AMPARs). The observed long latency of the glutamatergic neuronal response in slices is consistent with inhibition from GABA released by these early-responding interneurons. We therefore conclude that the early-responding cells in slices are GABAergic and hypothesize that, as in culture, their response is mediated by CP-KARs and CP-AMPARs.

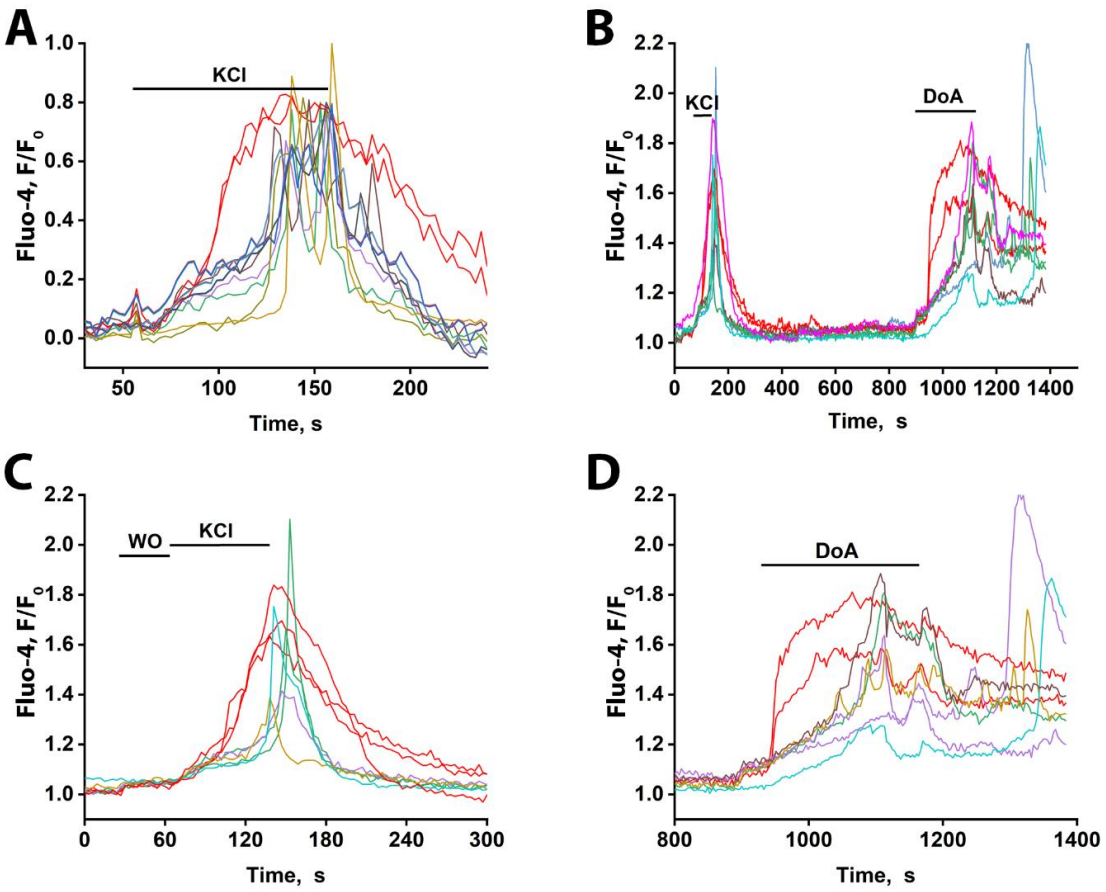

Figure 6. Ca^{2+} dynamics measured with Fluo-4 in slices of the CA1 region of the rat hippocampus in response to 50 mM KCl and 5 μM DoA. (**A**) - KCl-induced Ca^{2+} responses. Two neurons responded rapidly with a single, non-oscillatory $[Ca^{2+}]i$ increase (red traces); while the other neurons reacted after a 30-40 s delay, with oscillatory Ca^{2+} signals. In the field of view, 5 neurons showed the early response, and 50-60 cells showed the delayed, oscillatory response. On average delay between the onset of early and late responses was 40 s. (**B**) - $[Ca^{2+}]_i$ dynamics in individual neurons in response to 50 mM KCl and 5 μM DoA. Of the three neurons that responded to KCl with an early signal, two show fast response to DoA. (**C**) **-** Time-expanded response to KCl from Figure

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5B. 3 neurons reacted ahead of 30 s with a Ca^{2+} increase without oscillations. The remaining neurons reacted with a delayed oscillatory response. WO – washing out. (**D**) - Time-expanded response to 5μM DoA from Figure 5B. Two neurons responded by increasing their Ca^{2+} levels without oscillations, 80 s before the rest of the neurons were activated. The remaining neurons responded with an oscillatory reaction with a delay.

3. Discussion

The present work is devoted to demonstrating the existence of a leading reaction to excitation of GABAergic neurons in slices of the adult animal hippocampus and to investigating the role of GABAergic neurons expressing CP-AMPARs or CP-KARs in the early signal to glutamate receptor agonists and depolarization.

It is believed that the level of excitation of glutamatergic neurons is controlled by inhibitory neurons. However, for effective control, a feedforward response mechanism by inhibitory neurons to excitation is required. Evidence for such a feedforward response in GABAergic neurons has been demonstrated upon application of glutamate receptor agonists or depolarization [1,3].

Several studies have described a minor subtype of low-threshold spiking (LTS) interneurons in the hippocampus that provide feedforward inhibition [11]. These neurons are also considered low-threshold. The authors believe that the feedforward action of LTS interneurons is mediated by the response of T-type Ca^{2+} channels (which are activated at -55mV and cause slow calcium spikes). When DoA acts, initial excitation of GABAergic neurons does not occur, but $[Ca^{2+}]_i$ increases due to influx through the CP-KARs receptor, which induces GABA release. LTS interneurons constitute 4,5-15% of all GABAergic neurons in the hippocampus [25]. Whereas GABAergic neurons expressing CP-KARs and CP-AMPARs (according to our data, constitute more than half of GABAergic neurons in hippocampal culture [1]. Therefore, it can be argued that the vast majority of inhibitory neurons exhibit a feedforward response to excitation, using low-threshold CP-KARs and CP-AMPARs.

This study aims to demonstrate the existence of a rapid, feedforward response in adult hippocampal GABAergic neurons and to investigate the role of calcium-permeable AMPARs and KARs (CP-AMPARs/CP-KARs) in mediating this response.

Neural circuit stability requires inhibitory neurons to control glutamatergic excitation. For maximum effectiveness, this control must involve a feedforward response, where inhibitory neurons are among the first to be activated. Evidence for such a feedforward response in GABAergic neurons has been shown in culture models [1,3]. A known candidate for this role is the low-threshold spiking (LTS) interneuron, which constitutes 4.5-15% of GABAergic neurons and operates via T-type calcium channels [11,25]. While LTS interneurons has been implicated in feedforward inhibition via T-type calcium channels [11,25], this mechanism cannot account for the widespread rapid responses we and others have observed in culture [1,3].

In contrast, we propose a more widespread mechanism: GABAergic neurons expressing CP-AMPARs and CP-KARs, which our data indicate represent over half of the GABAergic population in culture [1]. In our proposed model, CP-KARs on GABAergic neurons are activated at resting potentials. This allows for direct calcium influx through receptors like CP-KARs upon agonist binding, triggering immediate GABA release without requiring prior depolarization. Therefore, we suggest that the predominant mechanism for feedforward inhibition involves CP-KARs, enabling a rapid response from the majority of inhibitory neurons and presents a fundamental and widespread mechanism for rapid inhibitory control.

GABAergic neurons in hippocampal culture can be classified into two subtypes based on their expression of either calcium-permeable KARs (CP-KARs) or CP-AMPARs [1,9,12]. These receptors are characterized by their high agonist affinity and sensitivity to depolarization, making them low-threshold sensors for excitation [1,3]. This property enables GABAergic neurons to generate a rapid, feedforward response to excitatory stimuli. Depolarization and agonist binding triggers a selective Ca^{2+} rise in these neurons, leading to immediate GABA release [5–7] and subsequent inhibition of downstream neuronal activity. The resulting delay in network excitation is primarily mediated by postsynaptic hyperpolarization via GABA(A) receptors, a conclusion supported by the abolition of

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the delay with the antagonist bicuculline (Fig. 2A). Thus, the feedforward inhibitory capability of these GABAergic neurons stems directly from the low-threshold properties of CP-KARs and CP-AMPARs.

The duration of the delay in glutamatergic neuron excitation varies significantly across the population (Figure 2B). This heterogeneity may be explained by differences in the chloride ion gradient among target neurons, which determines whether GABA(A) receptor activation results in hyperpolarization or depolarization [19]. Variations in this gradient would lead to differing degrees of GABA(A)R-dependent inhibition, thus accounting for the observed spectrum of delays and potentially contributing to neuronal desynchronization. Furthermore, the specific neural circuit involving the two GABAergic subtypes is critical. We previously showed that GABAergic neurons expressing CP-AMPARs innervate and inhibit those expressing CP-KARs [1,2,5]. Therefore, when NBQX blocks CP-AMPARs, it likely *disinhibits* the CP-KAR-expressing GABAergic neurons. This disinhibition enhances GABA release from these neurons onto their glutamatergic targets, thereby *increasing* the excitation delay (Figure 3A). This mechanism indicates that while both GABAergic subtypes respond early to DoA, the delay in glutamatergic excitation is predominantly controlled by the activity of CP-KAR-expressing neurons. This conclusion is supported by the observation that NBQX-mediated inhibition of CP-AMPARs, which removes a source of inhibition from the CP-KAR-expressing neurons, results in a longer delay.

The delay in glutamatergic excitation is primarily determined by the duration of GABAergic inhibition, as evidenced by its abolition with bicuculline. The latency is influenced by several factors, including the timing of GABA reuptake, individual neuronal chloride gradients, and the strength of the concurrent depolarizing current. DoA induces a biphasic response: an initial hyperpolarization via GABA release followed by a slow depolarization, likely mediated by calcium-impermeable AMPA receptors. Excitation occurs when this slow depolarization eventually reaches the threshold for sodium and calcium channel activation. Consequently, the net latency depends on the balance between the inhibitory GABAergic force and the magnitude of the slow depolarizing current, which is agonist concentration-dependent (Fig. 3C). This explains the inverse relationship between agonist concentration and delay duration observed in Figure 3; higher concentrations produce a stronger depolarizing current, overcoming the inhibition more quickly. A second mechanism influencing delay may involve a calcium-buffering system within CP-AMPAR-expressing GABAergic neurons. We propose that these neurons exhibit a delayed response until their intrinsic calcium-binding proteins are saturated. Once saturated, these neurons would then inhibit the CP-KAR-expressing GABAergic neurons. This *disinhibition* of the glutamatergic neurons targeted by CP-KAR cells, combined with the ongoing slow depolarization, would render them highly excitable and capable of generating low-amplitude, high-frequency oscillations. This represents a form of short-term plasticity that enhances the activity of a specific neuronal population.

Finally, the early-response property of neurons expressing CP-KARs/CP-AMPARs is not specific to DoA. As shown in Figures 4A, C, and D, these neurons also respond ahead of the main population to glutamate itself. Interestingly, a small subpopulation of glutamatergic neurons also exhibits an early response to glutamate (Figs. 4A, C). In contrast to the GABAergic neurons, however, these glutamatergic cells display asynchronous oscillatory responses.

To confirm the low-threshold excitability of GABAergic neurons expressing CP-KARs and CP-AMPARs, we applied a moderate depolarization (10-12 mV) using NH_4Cl. As shown in Figure 5A, both neuronal subtypes responded ahead of the general population, consistent with previous findings [1,3]. A key characteristic of these receptors is their low conductance, which allows subthreshold depolarization to elevate intracellular Ca^{2+} and trigger GABA release without necessarily generating action potentials.

We next tested neuronal responses under conditions of enhanced network excitability. Forskolin, a strong activator of adenylate cyclase, increases intracellular cAMP levels. This leads to PKA-dependent phosphorylation, which promotes depolarization by enhancing the activity of Ca^{2+} and Na^{+} channels while suppressing hyperpolarizing K^{+} channels. As expected, forskolin induced

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strong network-wide activation (increased firing frequency) in mature hippocampal cultures (Figure 5B). Under these conditions, a subset of neurons responded more rapidly. The subsequent application of ATPA (200 nM) induced a $[Ca^{2+}]_i$ increase specifically in these early-responding cells, identifying them as GABAergic neurons expressing CP-KARs. This rapid response to forskolin looks like the effect of other weak depolarizing agents [23,26], confirming that CP-KAR-expressing neurons are capable for early activation under various excitatory conditions.

To determine if this mechanism of excitation control operates in a more native context, we performed experiments on hippocampal slices from two-month-old animals. Figure 6 demonstrates that a small population of neurons in these slices exhibits a leading response to both depolarization and DoA, analogous to our culture findings. The same neurons responded more quickly to both stimuli, indicating the presence of an anticipatory GABAergic mechanism in vivo. This mechanism likely regulates hyperexcitability caused by depolarizing shifts and prevents the synchronization of glutamatergic networks.

4. Materials and Methods

4.1. Cell Culture Preparation

Primary neuronal-glial co-cultures were prepared from the hippocampi of neonatal Wistar rats (postnatal days 0–2). Briefly, after decapitation, hippocampal tissue was rapidly dissected into cold Versene solution, minced, and digested with 1% trypsin for 10 minutes at 37 °C under continuous agitation (~500 rpm). The digested tissue was then washed with chilled Neurobasal medium and mechanically dissociated by gentle trituration. The cell suspension was centrifuged at 2000 rpm for 3 minutes, and the pellet was resuspended in culture medium (Neurobasal medium supplemented with 2% B27, 0.5 mM glutamine, 1:250 penicillin-streptomycin). To optimize conditions for postnatal neurons, the NaCl concentration was adjusted to 4 g/L, matching that of Neurobasal-A medium.

For plating, 100 µL aliquots of the cell suspension were placed within glass cylinders (6 mm inner diameter, 7 mm height) positioned on polyethyleneimine-coated glass coverslips in Petri dishes. The cultures were incubated for 40 minutes (37°C, 5% CO_2, 95% humidity) to allow for cell attachment, after which the cylinders were carefully removed, and 2 mL of complete medium was added to each dish. Cultures were maintained with partial medium replacement (one-third volume) every four days and used for experiments after 13–14 days in vitro (DIV).

4.2. Hippocampal Slices

Hippocampal slices were obtained from two-month-old male Sprague-Dawley rats. The rats were subjected to terminal anesthesia by inhalation overdose of halothane, after which the brain was extracted and placed in ice-cold artificial cerebrospinal medium (aCSF) with the following composition: 124 mM NaCl, 26 mM $NaHCO_3$, 3 mM KCl, 2 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 1 mM $MgSO_4$, 10 mM glucose, saturated with a mixture of 95% O_2 and 5% CO_2 (pH 7.4) with 9 mM Mg^{2+}.

Coronal slices of the CA1 region of the hippocampus, 300 µm thick, were obtained using a Leica VT1200 vibratome. The obtained slices were incubated for 1 hour at room temperature in standard aCSF, saturated with a mixture of 95% O_2 and 5% CO_2.

Subsequently, the slices were transferred to a perfusion chamber and loaded with the fluorescent Ca^{2+}-sensitive probe Fluo-4 AM at a final concentration of 5 µM with the addition of 0.02% Pluronic F-127 for 1 hour. To complete the de-esterification of the probe, the slices were washed for 15 minutes in a perfusion system with aCSF saturated with a mixture of 95% O_2 and 5% CO_2.

After loading with the fluorescent probe, the slices were transferred to a specialized chamber for visualization—an RC-26G Open Diamond Bath Imaging Chamber (Warner Instruments)—and placed on the stage of a Zeiss LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany). An Achroplan 40x/0.8 W objective was used. The microscope settings for excitation and registration of Fluo-4 fluorescence were as follows: argon laser (488 nm), dichroic mirror HFT 488,

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emission filter BP 500–550 nm, gain 600–800, offset 0.1. The acquired image series were analyzed using ImageJ software (RRID: SCR_003070).

4.3. Fluorescent $[Ca^{2+}]_i$ Measurements

Intracellular calcium dynamics were monitored using the ratiometric indicator Fura-2 AM. Hippocampal cultures were incubated with 2–3 μM of the dye for 40 min at 28–37 °C in Hank's balanced salt solution (HBSS) containing (in mM): 136 NaCl, 3 KCl, 0.8 $MgSO_4$, 1.25 KH_2PO_4, 0.35 Na_2HPO_4, 1.4 $CaCl_2$, 10 HEPES, and 10 glucose, adjusted to pH 7.35. After loading, cells were washed several times with fresh HBSS to remove excess probe. Fluorescence was recorded on an inverted epifluorescence microscope (Leica DMI6000B, Leica Microsystems, Wetzlar, Germany) equipped with a Hamamatsu CCD camera and an external excitation filter wheel. Sequential illumination at 340 and 387 nm was provided, and emission was collected at 510 ± 40 nm using the FU2 filter set. Time-lapse imaging was performed at 28–30 °C in HBSS. Calcium signals were analyzed by calculating the 340/387 fluorescence ratio from regions of interest drawn over neuronal somata. Background signals obtained from cell-free areas of the field were subtracted before ratio calculation. The resulting traces represent relative changes in intracellular Ca^{2+} concentration.

4.4. Reagents

Domoic acid, NASPM trihydrochloride, ATPA, NBQX, forskolin (Tocris Bioscience, UK), bicuculline (Cayman Chemical, Ann Arbor, MI, USA), L-Glutamic acid (Sigma-Aldrich, Saint Louis, MO, USA), NH_4Cl (AppliChem, Darmstadt, Germany), Fura-2 AM (Molecular Probes, Eugene, OR, USA), Neurobasal-A medium (Life Technologies, Grand Island, NY, USA), B-27 supplement (Life Technologies, Grand Island, NY, USA), Trypsin 2.5% (Life Technologies, Grand Island, NY, USA).

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Abbreviations

GABA γ-aminobutyric acid
DIV days in vitro

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AP action potential

ATPA calcium-permeable kainate receptor agonist, (RS)-2-amino-3-(3-hydroxy-5-tertbutylisoxazol-4-yl) propanoic acid

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