Kainate induced ectopic spiking of pyramidal cells

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KA induced ectopic spiking of pyramidal neurons

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Abstract

Kainate receptors (KARs) are expressed at high levels in the brain during early development and may be critical for the proper development of neuronal networks. Here we have elucidated a physiological role of high-affinity KARs in developing hippocampal network by studying the effects of 25 - 100 nM kainate (KA) on intrinsic network activity in slice preparations. While 100 nM KA resulted in hyperexcitability of the network and the disruption of natural activity patterns, ≤ 50 nM KA concentrations enhanced the initiation of network bursts yet preserved the characteristic patterns of endogenous activity. This was not dependent on changes in GABAergic transmission or on activation of GluK1 subunit containing KARs. However, the activation of high-affinity KARs increased glutamatergic drive by promoting spontaneous firing of CA3 pyramidal neurons without affecting action potential independent glutamate release. This was not due to changes in the intrinsic somatic properties of pyramidal neurons but appeared to reside in an electrically remote site, most probably in an axonal compartment. While application of KAR agonists has mainly been used to study pathological type of network activities, the present study provides a novel mechanism by which endogenous activity of KARs can modulate intrinsic activities of the emerging neuronal network in a physiologically relevant manner. The results support recent studies that KARs play a central role in the activity-dependent maturation of synaptic circuitries.
Introduction

Kainate receptors (KARs) are a subfamily of ionotropic glutamate receptors thought to have an important role in the early development of central nervous system (Pinheiro and Mulle 2006). They are formed by heteromeric assembly of several subunits, which are expressed at particularly high levels with characteristic patterns of temporal regulation during early developmental stages (Bahn et al. 1994; Wilding and Huettner 2001; Lilliu et al. 2002; Ritter et al. 2002).

KARs have been implicated in various network functions in the brain. Kainate (KA) application is a potent way to generate and modulate rhythmic oscillations (Traub et al. 2004; Brown et al. 2006; Huxter et al. 2007), and to induce pathological synchrony in both in vivo and in vitro models of epileptogenesis (Tremblay and Ben-Ari 1984; Ben-Ari and Cossart 2000; Smolders et al. 2002). The suitability of KARs for modulation of network functions stems from their ability to influence both synaptic function and excitability of neurons (Pinheiro and Mulle 2006). Presynaptically, KARs act as auto- or heteroreceptors regulating transmitter release, dynamic properties of synapses (Schmitz et al. 2001; Lauri et al. 2001; Lauri et al. 2006) and axonal excitability (Kamiya and Ozawa 2000; Semyanov and Kullmann 2001; Maingret et al. 2005). Postsynaptic KARs contribute to synaptic transmission (Castillo et al. 1997; Vignes and Collingridge 1997; Cossart et al. 1998; Frerking et al. 1998) and modulate neuronal excitability by influencing intrinsic properties of neurons (Melyan et al. 2002; Ruiz et al. 2005; Segerstråle et al. 2010).

A characteristic feature of developing neuronal networks is intrinsic activity that is crucial for the synaptic maturation (Feller 1999; Ben-Ari 2001; Zhang and Poo 2001; Hanse et al. 2009). A prime example is the spontaneous network activity in the neonatal rodent hippocampus manifesting as synchronized population bursts, often referred to as Giant Depolarizing Poten-
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Recently, tonic activation of KARs containing subunit GluK1 (previously known as GluR5; Collingridge et al. 2009) by endogenous glutamate was shown to attenuate this bursting by regulation of glutamate release (Lauri et al. 2005). However, other physiological roles of KARs in network activity during early development remain unknown.

KAR subunits GluK4 and GluK5 (previously known as KA1 and KA2, respectively; Collingridge et al. 2009), which confer an especially high agonist affinity phenotype (Pinheiro and Mulle 2006, Jane et al., 2009), are highly expressed in the CA3 region of both immature and mature hippocampus (Werner et al. 1991; Herb et al. 1992; Bahn et al. 1994; Kask et al. 2000; Ritter et al. 2002). This suggests that high affinity KARs may have a physiologically important role in the neonatal hippocampus. In support for this, perinatal exposure to subconvulsive KAR agonist concentrations *in vivo* perturbs the neurobehavioral development in the rat (Doucette et al. 2003; Doucette et al. 2004), although the network and cellular basis for this are unknown.

While KAR agonists have previously been mostly used to study pathological types of hypersynchronous network activity, the current study was conducted to assess *in vitro* the physiological role of high-affinity KA receptors in the neonatal hippocampus. Of particular interest was the relation of high-affinity KARs to the endogenous network activity patterns critical for activity-dependent maturation of the hippocampal circuitry. It was observed that activation of high-affinity KA receptors leads to dramatic increase in occurrence of network bursts and glutamatergic drive in CA3 region, via increased generation of ectopic spikes in CA3 pyramidal neurons.
Materials and methods

Preparation of acute brain slices
Acute hippocampal slices were cut from the brain of postnatal day 3 to 6 (i.e. P3 to P6) Wistar rats using standard methods. Briefly, rats were killed by decapitation without anesthesia in accordance with the University of Helsinki animal welfare guidelines. A brain tissue block was dissected and glued onto the stage of a vibratome (Vibratome, www.vibratome.com), and 400 µm thick slices were cut parasagittally in an ice-cold solution containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 10 MgSO₄, 26 NaHCO₃, 15 D-glucose, and 1 CaCl₂ (saturated with 5% CO₂ / 95% O₂). Slices were stored at room temperature for at least one hour before recordings in a solution of following composition (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 4 MgSO₄, 26 NaHCO₃, 15 D-glucose, and 1 CaCl₂ (saturated with 5% CO₂ / 95% O₂).

Electrophysiological recordings
For recordings, slices were placed on a nylon mesh (blind setup) or glass plate (visualized setup) in a submerged recording chamber and superfused at a rate of 1-2 ml/min with artificial cerebrospinal fluid (aCSF) containing the following (in mM): 124 NaCl, 3 KCl, 1.25 NaH₂PO₄, 1 MgSO₄, 26 NaHCO₃, 15 D-glucose, and 2 CaCl₂ (saturated with 5% CO₂ / 95% O₂). Patch clamp recordings were made blindly from CA3 pyramidal neurons and under visual guidance from stratum (s.) lucidum interneurons. Interneurons were selected for recordings by visual identification under infrared illumination combined to differential interference contrast or Dodt gradient optics based on following criteria: (1) localization in the CA3 s. lucidum and (2) multipolar or bipolar shape of the cell soma, the latter in an orientation perpendicular to the pyramidal neurons. All experiments were done at 32 °C.
Electrodes were pulled from borosilicate glass capillaries (1.5/0.86 mm outer/inner diameter) with the Narishige PC-10 puller and had a resistance of 3-6 MΩ. For whole cell recordings of synaptic currents, neurons were voltage clamped at −70 to −75 mV. Electrodes were filled with filling solution I (composition in mM: 90 Cs-methanesulfonate, 30 CsCl, 10 HEPES, 10 EGTA, 4 ATP-Mg and 0.3 GTP-Na) for recordings of network bursts. Filling solution II (115 Cs-methanesulfonate, 10 HEPES, 10 EGTA or BAPTA, 4 ATP-Mg and 0.3 GTP-Na) was used for recordings of glutamatergic unitary currents. Intrinsic neuronal currents were recorded in whole cell current clamp configuration with filling solution III (110 K-gluconate, 20 KCl, 10 HEPES, 0.1 EGTA, 4 ATP-Mg and 0.3 GTP-Na). Spontaneous action currents were recorded in cell-attached configuration with aCSF-filled electrodes and amplifier set to "track" mode (Perkins 2006). The pH of filling solutions was adjusted to 7.20-7.30 with 1M CsOH or KOH. Osmolarities of solutions were 275-285 mOsm/l. All measured potentials were corrected for calculated liquid junction potentials (6-14 mV; Barry 1994). Mossy fiber responses were evoked by stimulating the granule cell layer of the dentate gyrus with a bipolar electrode.

Data were amplified and filtered (at 1-5 kHz) with Axopatch 200B (Molecular Devices [MD], www.moleculardevices.com) or Multiclamp 700A (MD) amplifier, digitized with Digidata 1322A (MD) and recorded (with sampling frequency ≥ 2.5 times the filtering frequency) using Clampex of pClamp 9 suite (MD). Uncompensated series resistance was monitored throughout recordings and measurements were excluded from analysis if it changed more than 20%.

**Data analysis**

Spontaneous events were detected with Clampfit of pClamp 9 suite. The threshold-based detection algorithm was used for network bursts and action currents and the template-matching algorithm for unitary synaptic currents. Detected events were verified visually. Current clamp
recordings were analyzed in Igor Pro (WaveMetrics, Inc.; www.wavemetrics.com) with Neuromatic module by Jason Rothman (www.neuromatic.thinkrandom.com). All average data is reported as mean ± standard error of mean (SEM) together with the number of recordings (n). Effects of drugs are quantified as a ratio over baseline conditions. Statistical significance of the difference between means was determined using unpaired or paired Student’s two-tailed T test. P-values less than 0.05 were considered statistically significant.

**Drugs**

All compounds were from Tocris or Sigma, except for LY382884, which was generously provided by Eli Lilly, Co.
Results

Low concentrations of KA promote initiation of network bursts at CA3 region

Spontaneous synaptic activity in pyramidal neurons of the neonatal hippocampal CA3 region (n = 39) was characterized by network bursts occurring on a background of asynchronous unitary postsynaptic currents (PSCs; Figure 1A). Average interval of the bursts was 11 ± 1 s and coefficient of variation (CV; i.e. standard deviation / mean) of burst interval 0.47 ± 0.03. Bursts had an average area (i.e. charge transfer) of 90 ± 10 pC, amplitude of 520 ± 70 pA and half-width of 130 ± 10 ms.

To study the effects of activation of KARs on network activity patterns, KA was bath-applied at nanomolar concentrations to the slices. As reported previously (Khalilov et al. 1999b), application of 100 nM KA resulted in a biphasic effect on network bursts: a transient decrease of burst interval down to 20% of baseline was followed by a complete disappearance of bursts, and their replacement by intense asynchronous activity (Figure 1A). In contrast, ≤ 50 nM KA resulted in a sustained increase of burst occurrence without disrupting the patterned nature of activity (Figure 1Aii). 25 nM KA decreased burst interval reversibly to 55 ± 6% (p < 0.01; n = 5) and 50 nM to 26 ± 8% of baseline (p < 0.05; n = 7; Figure 1B). No KA induced membrane currents were observed with concentrations below 100 nM (data not shown). During 50 nM KA application bursts also occurred in a more regular manner since the CV of interval decreased to 47 ± 8% of baseline (p < 0.01; Figure 1B) and burst amplitude decreased to 61 ± 9% of baseline (P < 0.05). When results from individual experiments with 25 and 50 nM KA were pooled, changes in burst amplitude and interval were highly correlated (r² = 0.60 for Pearson's correlation test, p < 0.001; Staley et al. 1998; Tabak et al. 2001).
Whilst showing a clear preference for KARs, kainate at higher concentrations may also activate AMPA receptors (Huettner 1990; Lerma et al. 1993; Wilding and Huettner 1996; Clarke et al. 1997). To ensure that the network effects of KA were indeed mediated by KAR activation, the experiments were repeated with domoate (DA), a more specific agonist for KARs (Huettner 1990). 5 nM DA (n = 6) decreased burst interval to 47 ± 7% (p < 0.001) and CV to 56 ± 5% of baseline (p < 0.001; Figure 1B), respectively. There was no significant change in burst size. The effect of DA was qualitatively similar to that of kainate, which indicates that the effects of kainate are solely due to activation of KARs with no apparent role for AMPA receptors.

Initiation of network bursts is a property of local circuits in all structures of neonatal hippocampus, although primary sites appear to be CA3 and dentate gyrus (DG; Strata et al. 1997; Menendez de la Prida and Sanchez-Andres 2000; Bolea et al. 2006). Present observations from CA3 region reveal that activation of high-affinity KARs have a marked effect on the mechanisms that generate bursts. To clarify whether this reflects increased burst initiation locally at CA3 or remotely at DG, we assessed the effects of KA on synaptic activity in the DG. In recordings from granule cells, unitary PSCs were virtually absent (data not shown), but network bursts occurred at an average interval of 16 ± 4 s and CV of interval of 0.52 ± 0.04 (n = 8). Burst area was 22 ± 8 pC, amplitude 125 ± 46 pA and half-width 75 ± 29 ms. 50 nM KA decreased burst interval to 53 ± 10% of baseline (p < 0.05; Figure 1C) with no change in burst variability. Amplitude decreased to 78 ± 8% (p < 0.05). In comparison to CA3, the effect on occurrence was significantly smaller (p < 0.05) at DG. To further examine the role of DG, network activity was recorded from slices with CA3 region surgically isolated from DG. In isolated CA3, average burst intervals were 21 ± 4 s with a CV of 0.59 ± 0.07 (n = 5). 50 nM KA had similar effects to that seen previously in intact slices on both burst interval and CV (n = 5; data not shown). Thus, burst interval and CV were 26 ± 9% (vs. 26 ± 8%
in intact slices; NS) and 56 ± 5% (vs. 47 ± 8%; NS) of baseline values, respectively. Together these findings indicate that enhanced bursting in KA reflects increased burst initiation at CA3 with negligible role of DG.

Gamma-aminobutyric acidergic (GABAergic) transmission is a critical factor underlying the generation of network activity patterns in the neonatal as well as in the adult hippocampus (see e.g. Lämsä and Taira 2003; Ben-Ari et al. 2007). To examine the role of fast GABAergic transmission in the effects of KA, network activity was recorded in the presence of GABA_A receptor blocker picrotoxin (100 µM). After ≥ 30 min block of GABA_A receptors, network generated interictal-like bursts (Figure 2A) occurring with an average interval of 23 ± 3 s and CV of interval of 0.54 ± 0.05 (n = 5) as reported previously (Khalilov et al. 1999a; Lämsä et al. 2000; Wells et al. 2000; Galindo et al. 2005). 50 nM KA decreased burst interval to 31 ± 9% (p < 0.05), CV of interval to 51 ± 7% (p < 0.01) and amplitude to 69 ± 9% of baseline (p < 0.05; Figure 2Bii). These effects did not differ from those in the presence of functional GABAergic transmission.

As GABA_B receptors are also able to modify the activity of neonatal hippocampus (McLean et al. 1996; Tosetti et al. 2004), the effects of KA were assessed in the presence of a GABA_B receptor blocker (2S)-3-[(1S)-1-(3,4-Dichlorophenyl)ethyl]amino-2-hydroxypropyl](phenylmethyl)phosphinic acid hydrochloride (CGP 55845; 1 µM). In CGP 55845, network bursts occurred with an interval of 41 ± 14 s and CV of interval of 0.67 ± 0.18. In every recording, some bursts were drastically prolonged (up to >20 s; data not shown) in accordance with a previous study (McLean et al. 1996). Subsequent co-application of 50 nM KA decreased burst interval to 34 ± 5% of baseline (p < 0.05) analogous to that seen in the absence of CGP55845 (NS; Figure 2B). However, KA did not significantly alter the regularity of bursts (CV of interval was 79 ± 28% of baseline; NS) in the presence of CGP 55845 (Figure 2B) probably because the temporal patterns of bursting are perturbed in the absence of GABA_B
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dependent inhibition (McLean et al. 1996). The consistency of KA's network effect under block of GABA_A and GABA_B receptors indicate that the mechanisms underlying the effects of KA are independent of GABA receptor mediated transmission.

KARs containing subunit GluK1 (GluK1-KARs) modulate the patterns of spontaneous network activity in neonatal hippocampus (Lauri et al. 2005). To see if they contribute to the network effects generated by low doses of KA, 50 nM KA was applied in the presence of a specific blocker of GluK1-KARs, LY382884 (10 µM; Figure 2C). In accordance with Lauri et al. (2005), LY382884 increased burst interval to 135 ± 13% of baseline (P < 0.05; n = 5) with no effect on CV of interval (Figure 2C). Also, LY382884 increased burst amplitude to 130 ± 8% of baseline (P < 0.05) in line with the correlation of burst size with interval (see above). Subsequent co-application of 50 nM KA decreased burst interval to 24 ± 4% of baseline (P < 0.01; n = 5), CV of interval to 64 ± 9% of baseline (Figure 2C) and amplitude to 66 ± 9% of baseline (P < 0.05). As these changes were similar to those in the absence of LY382884 (all differences were NS), the effects of low KA doses on temporal properties of network activity are not mediated by activation of GluK1-KARs.

In conclusion, ≤ 50 nM KA concentrations increase both occurrence and regularity of network bursts without inducing hypersynchronous network bursts as with higher KA concentrations. Taken together, these observations suggest that activation of high-affinity KARs enhances network burst initiation mechanisms at neonatal CA3 independently of GABAergic transmission and GluK1-KARs.

KA augments action potential dependent glutamatergic transmission

To address the effects of KA on glutamatergic transmission, AMPAR-mediated spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the presence of blockers of GABA_A (100 µM picrotoxin) and N-methyl-D-aspartic acid (NMDA) receptors (50 µM D-(-)-2-Amino-5-phosphonopentanoic acid [D-AP5]). In pyramidal neurons (n = 11), average sEPSC
interval was $5.8 \pm 2.3$ s, amplitude $30 \pm 5$ pA, rise time $1.7 \pm 0.2$ ms and decay time $5.8 \pm 0.8$
ms (Figure 3Ai). In interneurons ($n = 17$), interval was $2.3 \pm 1.1$ s, amplitude $31 \pm 5$ pA, rise
time $1.3 \pm 0.1$ ms and decay time $5.1 \pm 0.3$ ms (Figure 3Aii). 50 nM KA substantially in-
creased the occurrence of sEPSCs in both pyramidal neurons (Figure 3B) and interneurons
(figure 3C) in CA3. sEPSC interval decreased reversibly to $20 \pm 7\%$ (p < 0.05) and $24 \pm 4\%$
(p < 0.001) of baseline in pyramidal neurons and interneurons, respectively. However, no ef-
fect on either sEPSC amplitude or kinetics in pyramidal neurons (data not shown) or kinetics
in interneurons (data not shown) was observed. This suggests that there was no change in the
active afferent population, although, curiously, sEPSC amplitude increased reversibly to $153$
$\pm 20\%$ of baseline (P < 0.05; $109 \pm 15\%$ after washout) in interneurons.

Two major glutamatergic inputs to CA3 region are recurrent axons of CA3 pyramidal neu-
rons and mossy fiber axons from granule cells of dentate gyrus. These inputs can be pharma-
cologically differentiated as activation of group II metabotropic glutamate receptors (mGluR-
II) selectively suppresses mossy fiber transmission (Kamiya et al. 1996; Gutiérrez et al. 2003;
Kasyanov et al. 2004; Marchal and Mulle 2004; Safiulina et al. 2006). First, the effects of a
mGluR-II agonist $(2S,2'R,3'R)-2-(2',3'-$Dicarboxycyclopropyl)$glycine (DCG IV; 1 µM) on
PSCs evoked by stimulation of granule cells (eEPSCs) were assessed to ascertain the sensi-
tivity of neonatal mossy fibers to mGluR-II activation. DCG-IV suppressed the amplitude of
eEPSCs to $48 \pm 9\%$ and $36 \pm 9\%$ of baseline in pyramidal neurons ($n = 9$, p < 0.005) and in-
terneurons ($n = 11$; p < 0.001), respectively (data not shown). In contrast, DCG-IV caused no
change in sEPSC occurrence in either pyramidal neurons ($n = 6$; Figure 3Bi) or interneurons
($n = 10$; Figure 3Ci) nor affected their amplitude or kinetics (data not shown), indicating
that mossy fibers do not substantially contribute to ongoing unitary glutamatergic transmis-
sion at CA3. Upon 50 nM KA application in the presence of DCG-IV, sEPSC interval de-
creased to $19 \pm 6\%$ of baseline (p < 0.01) in pyramidal neurons (Figure 3Bi) and to $33 \pm$
10% of baseline (p < 0.005) in interneurons (Figure 3Ciii). There was no effect on amplitude in pyramidal neurons and an increase to 170 ± 29% of baseline (p < 0.05) in interneurons (data not shown; see also 3C). Thus, the effects of KA on sEPSCs were similar in the presence and absence of DCG IV, which indicates that spontaneous mossy fiber transmission is not enhanced by activation of high-affinity KARs.

KARs modulate transmitter release in several synapses (e.g. Cossart et al. 2001; Kidd et al. 2002; Delaney and Jahr 2002; Lauri et al. 2005). To address the effects of high-affinity KAR activation on action potential independent glutamate release, AMPAR-mediated miniature EPSCs (mEPSCs) were recorded in the presence of a blocker for voltage-gated sodium channels (tetrodotoxin [TTX]; 1 µM) in otherwise similar conditions as sEPSCs. In CA3 pyramidal neurons (n = 5) mEPSC interval was 5.9 ± 2.1 s, amplitude 39 ± 6.2 pA, rise time 2.5 ± 0.2 ms and decay time 8.8 ± 1.9 ms. In interneurons (n = 12) mEPSC interval was 5.0 ± 1.2 s, amplitude 32 ± 3 pA, rise time 1.5 ± 0.1 ms and decay time 5.1 ± 1.0 ms. Upon application of 50 nM KA there was no significant change in interval (Figure 4), amplitude or kinetics (data not shown) of mEPSCs in either cell type.

These observations show that activation of high-affinity KARs leads to marked increase in action potential dependent glutamatergic transmission in CA3 region of neonatal hippocampus. As this effect does not appear to involve a significant contribution by mossy fibers, it seems to reflect increased activity of local axons in the CA3 region. One possible mechanism to account for these observations would be a KAR-mediated modulation of CA3 pyramidal neuron excitability.

**KA increases spontaneous spiking of CA3 pyramidal neurons**

KARs are able to regulate the activity of hippocampal neurons by affecting their intrinsic properties (Rovira et al. 1990; Nistri and Cherubini 1991; Melyan et al. 2002; Fisahn et al. 2005).
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289 2005; Ruiz et al. 2005). To address the effects of KA on somatic excitability of neurons, responses to depolarizing current steps (300 ms) were recorded from CA3 pyramidal neurons and interneurons with potassium-based electrode filling solution under current clamp in the presence of picrotoxin (100 µM), D-AP5 (50 µM) and an α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptor blocker GYKI53655 (50 µM). 50 nM KA had no effect on the response to depolarizing current steps in either neuron type (Figure 5), indicating that activation of high-affinity KARs does not increase somatic excitability of neurons at neonatal CA3.

297 KAR mediated depolarizing inward currents may also directly excite neurons (Cossart et al. 1998; Frerking et al. 1998), and CA3 pyramidal neurons are especially sensitive to this action (Robinson and Deadwyler 1981; Khalilov et al. 1999b). However, we found that 50 nM KA induced no change in membrane potential in pyramidal neurons clamped at -70 – 75 mV (1.6 ± 2.1 mV; NS) Nevertheless, even electrotonically remote depolarization could theoretically be manifested on the network level by adding up through neuron population. Therefore, the consequences of membrane depolarization for network function and cellular properties were addressed by elevating extracellular potassium concentration ([K+]o). On the network level, elevation of [K+]o by 1, 3 and 5 mM from 3 mM baseline value resulted in decrease of burst interval to 45 ± 5% (n = 3, p < 0.05), 22 ± 2% (n =5, p < 0.001) and 14 ± 2% (n =4, p < 0.001; data not shown). Thereby, approximately 2-3 mM elevation of [K+]o mimics the network effect of 50 nM KA (which decreased the burst interval to 26 ± 8% of baseline; 1B) on burst occurrence.

309 In current-clamped pyramidal neurons (n = 6), elevation of [K+]o by 3 mM and 5 mM resulted in 10.0 ± 1.8 mV and 10.0 ± 2.5 mV (p < 0.01) depolarization of membrane potential, respectively. In addition, 3 and 5 mM elevation of [K+]o decreased input resistance by 82 ± 26 MΩ (p < 0.05) and 118 ± 25 MΩ (p < 0.01), respectively, from 529 ± 70 MΩ (data not
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Thus, whereas 50 nM KA dramatically increased the burst occurrence without depolarizing the neurons, the elevation of [K\(^+\)]\(_o\) that mimics the effect of KA on network burst occurrence also induces a prominent somatic depolarization of the CA3 pyramidal neurons.

In light of these observations, the increase in sEPSC frequency by 50 nM KA cannot be explained by changes in excitability or passive membrane properties at the soma of CA3 pyramidal neurons. However, the lack of detectable inward currents in somatic recordings does not necessarily imply their absence from electrotonically remote locations of neurons. Indeed, KAR mediated depolarization may induce spike generation locally in interneuronal axons (Semyanov and Kullmann 2001). To study spontaneous generation of action potentials, activity of intact neurons was monitored by cell-attached recordings in the presence of picrotoxin (100 µM), D-AP5 (50 µM) and GYKI53655 (50 µM) to block fast synaptic input. Under these conditions, ~40% of recorded pyramidal neurons and ~60% of interneurons displayed spontaneous spiking. Spikes occurred at an average interval of 8 ± 4 s (n = 17) in pyramidal neurons (Figure 6A) and 1.6 ± 1.4 s (n = 6) in interneurons (not shown). Upon KAR application spike interval decreased reversibly to 30 ± 9% of baseline (n = 8; p < 0.01; Figure 6A and B) in pyramidal neurons but was unaffected in interneurons (97 ± 9% of baseline, NS; Figure 6C). However, in the absence of blockers application of 50 nM KA decreased spike interval in interneurons to 63 ± 13% of baseline value of 0.6 ± 0.2 s (n = 6, p < 0.05; Figure 6C). Increased spiking is most likely due to increased glutamatergic input to interneurons and further emphasizes the glutamatergic nature of the consequences of high-affinity KAR activation. These observations indicate that activation of high-affinity KARs enhances spontaneous generation of action potentials in pyramidal neurons, which offers a valid explanation for enhancement of both glutamatergic drive as well as network burst initiation.

KARs regulating glutamate release in the neonate hippocampus are tonically activated by endogenous glutamate even in the absence of ongoing network activity (Lauri et al., 2005,
To assess whether KARs regulating pyramidal neuron activity were endogenously active, the effects of AMPA/KAR blocker NBQX (50 µM; n = 8) on spontaneous spiking were studied. Upon application of NBQX a decrease in spike occurrence was seen (increase in spike interval to 162 ± 64% of baseline (p < 0.05; Figure 6B). This indicates that KARs regulating spiking activity of CA3 pyramidal neurons are endogenously activated. However, as the recordings were conducted under abolished synaptic transmission, the activation profile of KARs may differ significantly in physiological conditions with ongoing glutamatergic activity.
Discussion

KA has been extensively used as a research tool in the study of epileptic activity and network oscillations (Ben-Ari and Cossart 2000; Fisahn 2005). However, the specific physiological roles of KA receptors in generation and modulation of network activity are only beginning to be understood. The present study shows that low doses of KA, activating a subpopulation of high-affinity receptors, have a very specific influence on spiking activity of CA3 pyramidal neurons in the neonatal hippocampus. This action is central in the initiation of network bursts at the immature CA3 circuitry. Similar bursts are generated spontaneously in the neonate hippocampus and are critical for proper development of the synaptic connectivity (Lauri et al. 2003; Colin-Le Brun et al. 2004). Thus, high-affinity KARs may act as a physiological mechanism to trigger bursting activity in the immature network and thereby have a strong influence on the function and maturation of the whole hippocampal network.

KAR dependent initiation of network bursts

Activation of KARs by \( \leq 50 \) nM KA enhanced the natural activity patterns rather than induced seizure-like activity as does \( \geq 100 \) nM KA (Khalilov et al. 1999b; this study). This qualitative difference of effects suggests that lower nanomolar KA concentrations activate only a limited population of KARs with higher agonist affinity. As direct somatic depolarization of pyramidal neurons is central for drastic network effects of \( \geq 100 \) nM KA (Mulle et al. 1998; Khalilov et al. 1999b), lack of depolarization by \( \leq 50 \) nM KA provides a cellular level explanation for the concentration dependency of effects. In the absence of specific pharmacological tools, it is not feasible to directly test whether activation of these receptors by endogenous agonists is physiologically utilized for regulation of network function, but this possibility seems reasonable given the high ambient glutamate levels in immature brain tissue.
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(Danbolt 2001; Syková 2004; Lauri et al. 2006) as well as the sensitivity of the pyramidal cell spontaneous spiking to KAR antagonism.

In addition to burst occurrence, 50 nM KA increased burst regularity. Initiation of population bursts in the CA3 region of hippocampus is a threshold phenomenon depending on the attainment of adequate level of excitatory drive to trigger generation of a burst (Menendez de la Prida and Sanchez-Andres 1999a; Menendez de la Prida and Sanchez-Andres 1999b; Menendez de la Prida et al. 2006). Theoretically, burst occurrence could be increased by accelerated attainment or lowering of the burst initiation threshold. Because the temporal characteristics of bursting are mostly dictated by the mechanism driving the network towards threshold (Tabak et al. 2001; Agmon and Wells 2003), enhanced attainment of threshold would increase burst regularity whereas lowering of the burst initiation threshold would not necessarily do so. Therefore, increased burst regularity implies that KAR activation leads to enhancement of burst initiation mechanisms (Tabak et al. 2001; Agmon and Wells 2003).

Further support for the involvement of KARs in the burst initiation process is given by the barely evident effect of 50 nM KA on network activity in dentate gyrus. Although all structures of neonatal hippocampus are capable of burst generation, bursts are preferentially initiated in the CA3 region while dentate gyrus appears to serve mostly as a follower region (Menendez de la Prida and Sanchez-Andres 1999a; Lämsä et al. 2000; Menendez de la Prida and Sanchez-Andres 2000; Ben-Ari 2001; Bolea et al. 2006). The observed slight increase in burst occurrence in the dentate gyrus during 50 nM KA exposure could then be a manifestation of enhanced burst generation in the CA3. Independency of KA's network effects on GABA transmission is also compatible with the involvement of high-affinity KARs as GABA transmission is not absolutely required for burst initiation (Khalilov et al. 1999a; Lämsä et al. 2000).
Cellular mechanisms

Low burst initiation threshold of the CA3 region is due to the dense interconnections of local pyramidal neurons through recurrent axons (Miles and Wong 1983), and occurrence of network bursts is sensitive to changes in the glutamatergic drive in the neonatal network (Bennari et al. 1989; Bolea et al. 1999; Lauri et al. 2005). Consequently, increased glutamatergic drive at CA3 provides a plausible explanation for augmented initiation of network bursts.

Accordingly, application of 50 nM KA dramatically increased the occurrence of sEPSCs. The temporal properties of sEPSCs were regulated similarly in pyramidal neurons and interneurons, which suggests that effects were on a common afferent population. In addition, afferents appeared to remain the same during KA exposure, because sEPSC kinetics were unaffected. Mossy fiber input, assessed by sensitivity to mGluR-II agonists DCG IV, did not contribute to spontaneous glutamatergic drive at this developmental stage. Moreover, since DCG-IV did not influence the effects of KA, the active afferent population most probably consists of axons of local pyramidal neurons within area CA3.

KA receptors containing subunit GluK1 modulate the patterns of network activity in the neonatal hippocampus by regulation of action potential independent glutamate release (Lauri et al. 2005). Activation of high-affinity KA receptors had minor or no effects on mEPSCs, indicating that the KAR population involved in burst initiation is distinct from the previously characterized GluK1 containing receptor population. Since action potential independent glutamate release was not affected by 50 nM KA, the increase in glutamatergic drive must be due to increase in action potential generation in CA3 pyramidal neurons. Indeed, 50 nM KA dramatically increased spontaneous action potential firing of pyramidal neurons, which readily explains the augmented glutamatergic transmission and consequently enhanced network burst initiation.
Enhanced spiking of CA3 pyramidal neurons was not due to changes in somatic excitability or direct excitation by KA, although similar concentrations have been shown to enhance the excitability of mature pyramidal neurons in both CA3 (Rovira et al. 1990; Ruiz et al. 2005) and CA1 (Nistri and Cherubini 1991; Melyan et al. 2002; Fisahn et al. 2005), and, furthermore, such CA3 pyramidal neurons are clearly depolarized with KA concentrations as low as 100 nM (Robinson and Deadwyler 1981). This is in good agreement with the previous observations of Khalilov et al. (1999b) showing that susceptibility of CA3 pyramidal neurons to depolarizing action of KA develops gradually during first postnatal weeks. However, the lack of ionotropic effects with 50 nM KA in somatic recordings does not rule out this kind of action in an electrically distant location. In fact, in axons of hippocampal interneurons activation of KARs is able to induce antidromic spiking through depolarization of the axonal membrane (Semyanov and Kullmann 2001; Maingret et al. 2005) and at mossy fibers, KA reduces the threshold for antidromic spikes (Kamiya and Ozawa 2000; Schmitz et al. 2000). Thus, a possible explanation for increased ectopic spiking of CA3 pyramidal neurons could be an increased axonal excitability possibly by depolarization or modulation of axonal ionotropic properties (Vervaeke et al. 2006). Dynamic activation of this novel KAR dependent mechanism during enhanced glutamatergic activity provides powerful mechanism driving burst initiation in the immature circuitry.

**Implications and conclusions**

By efficient modulation of network burst initiation, high-affinity KA receptors may contribute to physiological activity patterns critical for activity-dependent maturation of the hippocampal circuitry. Maturation of hippocampal synapses and connections depends on electrical activity (Zhang and Poo 2001; Lauri et al. 2003; Colin-Le Brun et al. 2004; Spitzer 2006; Swann et al. 2007), and changes in network activity influence the modulation and balance of developing synapses (Kasyanov et al. 2004; Huupponen et al. 2007). The strong influence of
nanomolar KA on network activity in the developing hippocampus may well explain the changes in neurobehavioral development and hippocampal morphology produced by sub-convulsive doses of KAR agonists (Doucette et al. 2003; Doucette et al. 2004; Burt et al. 2008). In fact, present observations also have implications to neurobehavioral disturbances related to widely consumed stimulants ethanol and nicotine, that are both known to affect development (Berman and Hannigan 2000; Dwyer et al. 2009) and endogenous activity (Maggi et al. 2001; Galindo et al. 2005; Le Magueresse et al. 2006) of immature hippocampus. In addition, ethanol directly influences KAR function (Weiner et al. 1999; Costa et al. 2000). To conclude, this study revealed a novel, physiologically relevant role for high-affinity KA receptors in the generation of natural type of network activity in the neonatal hippocampus. By regulating the activity of CA3 pyramidal neurons, this sub-population of KA receptors can efficiently modulate glutamatergic drive in the CA3 region and thereby the occurrence of patterned activity critical for the proper development of the neonatal hippocampus. The finding that distinct KA receptor populations control the physiological patterns of network bursts and epileptiform activity may provide basis for development of pharmacological tools for treatment of neonatal hippocampal activity disorders.
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Figure legends

Figure 1 Low KAR agonist doses promote generation of network bursts

A Sample traces from voltage clamped (−70 mV) CA3 pyramidal neurons illustrating the effects of KA for network activity. i, During application of 100 nM KA, transient increase in the occurrence of network bursts was followed by intense asynchronous unitary activity (P4). ii, 50 nM KA induced sustained increase in network bursting (P5). Example bursts marked with asterisks are shown with an extended time scale on the right. Recordings were made with the filling solution I containing 30 mM chloride. B i, A summary of relative occurrence of network bursts in CA3 pyramidal neurons illustrating the temporal profile of the effect of 25 (n = 5), 50 (n = 7) and 100 nM (n = 4) KA as well as 5 nM DA (n = 6). Note that DA values are shifted 1 min to the right to reveal the 25 nM KA data points. ii, Average changes from baseline of burst interval and CV of interval in the presence of 25 nM and 50 nM KA as well as 5 nM DA. C Similar data as in B for the effects of 50 nM KA on network bursts recorded from granule cells of dentate gyrus. (In this and subsequent figures, asterisks denote P-values for difference to baseline with paired Student’s T-test: * stands for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$.)

Figure 2 Network effect of KA is not dependent on GABAergic transmission or GluK1 containing KARs

A Example traces from a voltage clamp recording of a CA3 pyramidal neuron (P5) in the presence of 100 µM picrotoxin illustrating the properties of GABAA receptor independent bursts and the effect of 50 nM KA on their occurrence. Example bursts marked with asterisks are shown with an extended time scale on the right. B Pooled data of relative occurrence (i) and changes from baseline of interval and CV of intervals (ii) of GABAA (n = 5) and GA-
BAB (n = 5) receptor independent bursts in 50 nM KA. C Similar data (n = 5) as in B for effects of 10 µM LY382884 and co-applied 50 nM KA on relative occurrence of network bursts in CA3 pyramidal neurons.

**Figure 3 KA enhances spontaneous glutamatergic transmission**

A Sample traces from a CA3 pyramidal neuron (P5, i) and a s. lucidum interneuron (P5, ii) illustrating the effect of 50 nM KA on occurrence of sEPSCs. Cells were voltage clamped at –75 mV with filling solution II, and recordings were made in the presence of 100 µM picrotoxin and 50 µM D-AP5. B Summaries of the effects of 50 nM KA on sEPSCs occurrence (i and ii) in pyramidal neurons (n = 5). Summary of effects of DCG IV (1 µM) and subsequently co-applied 50 nM KA on sEPSC interval in CA3 pyramidal neurons (n = 6, iii). C Similar data as in B for interneurons (n = 7 for i and ii; n=10 for iii).

**Figure 4 KA has no effect on action potential independent glutamatergic transmission**

A Sample traces (i) and pooled data of the effects of 50 nM KA on the occurrence (ii and iii) of mEPSCs in CA3 pyramidal neurons (n = 5). B Similar data as in A for s. lucidum interneurons (n = 12). Cells were voltage clamped at -75 mV with filling solution 2. Recordings were made in the presence of 100 µM picrotoxin, 50 µM D-AP5 and 1 µM TTX.

**Figure 5 KA does not affect intrinsic somatic properties of neonatal neurons**

Sample traces and pooled data of the effects of 50 nM KA on action potential firing in response to depolarizing current injections of 300 ms in CA3 pyramidal neurons (Ai and Aii, n = 5) and s. lucidum interneurons (Bi and Bii, n = 4). Summaries of the effects of 50 nM KA on input resistance and on injected current required to hold membrane potential at around –75
mV in CA3 pyramidal neurons (Ai) and interneurons (Bi). Recordings were made in the presence of 100 µM picrotoxin, 50 µM GYKI53655 and 50 µM D-AP5.

**Figure 6 KA increases spontaneous spiking of CA3 pyramidal neurons**

A Sample traces from a cell-attached recording of a CA3 pyramidal neuron (P4) illustrating the spontaneous occurrence of action currents and increased spiking in 50 nM KA. Events marked with asterisks are shown with an extended time scale on the right illustrating typical action current waveform and occurrence of spikes in clusters. Recordings were made with aCSF filled electrodes in the presence of 100 µM picrotoxin, 50 µM GYKI53655 and 50 µM D-AP5. B, Summary of the effects of 50 nM KA (n = 8) as well as block of KARs with 50 µM NBQX (n = 8) on relative occurrence (i) and average interval (ii) of spontaneous action currents. C, Similar summary as in B of the effects of 50 nM KA on spiking of interneurons in the presence (n = 6) and absence (n = 6) of blockers of synaptic transmission.
**A**

i. Baseline

100 nM KA

washout

10 s

400 pA

**B**

i. Kainate

Relative occurrence

0 10 20 30 40

time (min)

25 nM KA

50 nM KA

100 nM KA

5 nM DA

**C**

i. Kainate

Relative occurrence

0 10 20 30 40

time (min)

**ii.**

Change in CV of interval

2.5

treatment wash

25 nM KA 50 nM KA 5 nM DA

**ii.**

Change in CV of interval

2.5

treatment wash

25 nM KA 50 nM KA 5 nM DA
A

baseline

kainate

washout

B

i

kainate

- GABA_A block
- GABA_B block

ii

- GABA_A block
- GABA_B block

C

i

LY382884

kainate

ii

- LY
- LY+KA

wash

- LY
- LY+KA

wash
A

i

baseline

kainate

washout

5 s

20 pA

ii

relative occurrence

0 1 2 3

time (min) 0 10 20 30 40

kainate

iii

interval change

0 1 2

KA wash
A

baseline

kainate

washout

B

i

relative occurrence

KA

NBQX

interval change

KA

wash

ii

C

i

kainate

relative occurrence

in blockers

no blockers

interval change

KA

wash

ii