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Activation of κ opioid receptors increases intrinsic excitability of dentate gyrus granule cells

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Non-technical summary The hippocampus is an area of the brain that is important for learning and memory and a locus for hyperexcitable activity, such as epilepsy. The dentate gyrus (DG) of the hippocampus controls information flow into the rest of the hippocampus, and thus provides protection from excess activity. Under pathological conditions, such as epilepsy, this protective feature is circumvented and uninhibited activity flows throughout the hippocampus. Activation of kappa (κ) opioid receptors (KORs) prevents both the behavioural and electroencephalographic measures of seizures in several models of epilepsy, and other complex behaviours such as stress-induced deficits in learning. Understanding the cellular effect of KOR activation is critical to our understanding of DG function. In this study we show that, contrary to our hypothesis, activation of the KORs increases excitability of the DG cells. These results suggest that regulation of activity in the DG by activation of KORs is more complex than previously thought.

Abstract The dentate gyrus of the hippocampus is thought to control information flow into the rest of the hippocampus. Under pathological conditions, such as epilepsy, this protective feature is circumvented and uninhibited activity flows throughout the hippocampus. Many factors can modulate excitability of the dentate gyrus and ultimately, the hippocampus. It is therefore of critical importance to understand the mechanisms involved in regulating excitability in the dentate gyrus. Dynorphin, the endogenous ligand for the kappa (κ) opioid receptor (KOR), is thought to be involved in neuromodulation in the dentate gyrus. Both dynorphin and its receptor are widely expressed in the dentate gyrus and have been implicated in epilepsy and other complex behaviours such as stress-induced deficits in learning and stress-induced depression-like behaviours. Administration of KOR agonists can prevent both the behavioural and electroencephalographic measures of seizures in several different models of epilepsy. Antagonism of the KORs also prevents stress-induced behaviours. This evidence suggests the KORs as possible therapeutic targets for various pathological conditions. In addition, KOR agonists prevent the induction of LTP. Although there are several mechanisms through which dynorphin could mediate these effects, no studies to date investigated the effects of KOR activation on intrinsic membrane properties and cell excitability. We used whole-cell, patch-clamp recordings from acute mouse hippocampus slices to investigate the effect of KOR activation on dentate gyrus granule cell excitability. The agonist U69,593 (U6, 1 μM) resulted in a lower spike threshold, a decreased latency to first spike, an increased spike half-width, and an overall increase in spike number with current injections ranging from 15 to 45 pA. There was also a reduction in the interspike interval (ISI) both early and late in the spike train, with no change in membrane potential or input resistance. Preincubation of the slice with the selective KOR antagonist, nor-binalthorphimine (BNI, 1 μM) inhibited the effect of U6 on the latency to first spike and spike half-width suggesting that these effects are mediated through KORs. The inclusion of GDP-βS (1 mM) in the recording pipette prevented all of the U6 effects, suggesting that all effects are mediated via a G-protein-dependent mechanism. Inclusion of the A-type K^+ current blocker, 4-aminopyridine (4-AP, 5 mM) in the

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pipette also antagonised the effects of U6. Kv4.2 is one of the channel α subunits thought to be responsible for carrying the A-type K^+ current. Incubation of hippocampus slices with U6 resulted in a decrease in the Kv4.2 subunit protein at the cell surface. These results are consistent with an increase in cell excitability in response to KOR activation and may reflect new possibilities for additional opioid functions.

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Abbreviations BNI, nor-binalthorphimine; DG, dentate gyrus; ISI, interspike interval; KOR, κ opioid receptor; LTP, long-term potentiation; U6, U69,593.

Introduction

Dynorphin is a member of the opioid family and is the endogenous ligand of the κ opioid receptor (KOR) (Chavkin *et al.* 1982). Several different dynorphin molecules (dynorphin A, dynorphin B, and big dynorphin) have been discovered which are all derived from a common precursor, prodynorphin (Kakidani *et al.* 1982; Watson *et al.* 1983) and have a binding preference for the KOR (Chavkin *et al.* 1982). Big dynorphin includes dynorphin A, the first 17 amino acids on the amino-terminus and dynorphin B on the carboxy-terminus (Fischli *et al.* 1982). The distribution of these different dynorphins is similar throughout the nervous system (Watson *et al.* 1983; Chavkin *et al.* 1985); however, they have varying potencies at the KOR (James *et al.* 1984).

Activation of the KORs in the hippocampus is involved in complex pathological behaviours including stress-induced behaviours and epilepsy. Stress activates the KOR system in the hippocampus (McLaughlin *et al.* 2003*a*). A number of different stress-inducing procedures increase the levels of dynorphins in the hippocampus. Dynorphin A levels increase after immobilisation, forced swim and learned helplessness specifically in the hilus of the dentate gyrus (DG) and in the mossy fibre pathway (Shirayama *et al.* 2004). Immobilisation and learned helplessness also increase levels of dynorphin B in the mossy fibre pathway of the DG (Shirayama *et al.* 2004). In addition, activation of the KOR mediates stress-induced deficits in learning and memory (Carey *et al.* 2009). Depression is a serious and common consequence of chronic stress, and recent data also suggest that the KOR system is responsible for stress-induced depression-like behaviours such as place aversion in rodents (Land *et al.* 2008). These aversive behaviours can be triggered by the administration of KOR agonists alone (Shippenberg & Herz, 1986; Suzuki *et al.* 1992; Bruchas *et al.* 2007; Land *et al.* 2008), and they are absent in mice lacking the prodynorphin gene (Land *et al.* 2008).

Convincing evidence suggests that κ opioids are involved in controlling excitability in the central nervous system. Dynorphin release in the hippocampus, as measured by microdialysis, is increased during seizures induced by amygdaloid kindling in rats (Rocha *et al.* 1997). On the other hand, dynorphin protein expression is reduced in hippocampus tissue following seizures induced by amygdaloid kindling (McGinty *et al.* 1986; Romualdi *et al.* 1995). Seizures induced by kainic acid (KA) also result in a reduction in dynorphin immunoreactivity in the hippocampus of rats 6 h after KA administration (Kanamatsu *et al.* 1986), and also in mice 4 days after KA (Gall, 1988). The paradox of the increased release followed by a decrease in protein expression is thought to be caused by enhanced dynorphin release and, possibly, depletion during the seizures. Expression of prodynorphin mRNA is increased in the hippocampus of rats, for at least 24 h after a seizure induced by KA (Douglass *et al.* 1991; Lason *et al.* 1992) and at least 60 min after a seizure induced by amygdaloid kindling (Romualdi *et al.* 1995), possibly to account for the protein depletion during seizures. It is thought that dynorphin released during seizures acts to limit the spread of excitability, and there is a significant amount of evidence to suggest that it acts as an anticonvulsant. KOR-specific agonists provide a protective effect against seizures in electrically induced rat epilepsy models (Tortella *et al.* 1986, 1989, 1990), chemically induced seizure models (Tortella *et al.* 1990; Przewlocka *et al.* 1994), and a virally induced seizure model (Solbrig *et al.* 2006). In addition, prodynorphin knockout mice display lower thresholds for seizure induction by kainic acid or the $GABA_A$ antagonist pentylenetetrazole (Loacker *et al.* 2007).

Within the hippocampus, the DG shows the most concentrated staining for dynorphin. Both dynorphin A and dynorphin B are localised to the DG. In rats, dynorphin A immunoreactivity is most prominent in the mossy fibre projection from the granule cells of the DG (McGinty *et al.* 1983; Chavkin *et al.* 1985), while dynorphin B is heavily concentrated in DG granule cells and their projections, and in multipolar neurons scattered in the granule and molecular layers (Chavkin *et al.* 1985). Dynorphin A and B immunoreactivity in the mouse was similar to that in the rat, primarily localised to the mossy fibre system (Gall, 1988). Consistent with this distribution,

the mRNA for prodynorphin is most prominent in the granule cell layer of the mouse DG (Lin *et al.* 2006).

KORs are also localised to the hippocampus, although they show some variability in distribution and affinity between species (McLean *et al.* 1987). Radiolabelled ligands were used to identify a high affinity KOR in the molecular layer of the DG of guinea pigs (κ_1) (Zukin *et al.* 1988; Wagner *et al.* 1992) and a low affinity KOR in the dentate granule cell layer of rats (κ_2) (Zukin *et al.* 1988). Antisera raised against the cloned KOR confirm its presence in the granule cell layer and portions of the molecular layer in guinea pig (Drake *et al.* 1996).

The DG granule cells release dynorphin in two locations: the mossy fibre terminals (Gall *et al.* 1981; McGinty *et al.* 1983; McLean *et al.* 1987; Wagner *et al.* 1991) and the granule cell dendrites in the molecular layer of the DG (Drake *et al.* 1994; Simmons *et al.* 1995). Synaptic release of dynorphin at the mossy fibre terminals in acute hippocampus slices from guinea pig causes an inhibition of glutamate release from the presynaptic terminal at surrounding mossy fibres and subsequently an inhibition of long-term potentiation (LTP) at the neighbouring mossy fibre–CA3 synapses (Weisskopf *et al.* 1993). Exogenous application of dynorphin also decreases mossy fibre responses in acute hippocampus slices from mice, hamsters and Long-Evans rats (Salin *et al.* 1995).

Activation of KORs at the perforant path–granule cell synapse has a similar effect to those at the mossy fibre–CA3 synapse. Application of U6, a selective KOR agonist, to acute guinea pig hippocampus slices inhibits glutamate release from perforant path synapses (Simmons *et al.* 1994), which reduces synaptic transmission (Wagner *et al.* 1992), and inhibits the induction of LTP (Terman *et al.* 1994). Endogenously released dynorphin also has the effect of reducing synaptic transmission and blocking LTP induction at this synapse (Wagner *et al.* 1993; Terman *et al.* 1994). KOR activation is also thought to enhance excitability in the DG by inhibiting interneurons and thereby disinhibiting dentate granule cells (Neumaier *et al.* 1988).

The data describing the actions of κ agonists on cell membrane excitability are less clear. In CA1 cells from acute rat hippocampus slices, application of low concentrations of dynorphin A increased the M-current (Madamba *et al.* 1999). The M-current (I_M) is a potassium current that is active around resting membrane potentials and acts to dampen neuronal activity. Thus the effect of dynorphin A on CA1 cells has the consequence of decreasing cell excitability. Neither U6 nor U50,488 (U50) (selective KOR agonists) had a consistent enhancing effect on the I_M in these same cells, which the authors suggest is due to actions at different KOR subtypes (Madamba *et al.* 1999). In CA3 cells, both dynorphin A and U50 were found to augment I_M (Moore *et al.* 1994). At higher concentrations, dynorphin A was found to reduce I_M, an effect attributable to a loss of selectivity for the KOR and activation of other opioid receptor subtypes (Moore *et al.* 1994). Surprisingly, despite the finding that within the hippocampus KORs are most abundant in the DG, the effects of KOR activation on dentate granule cell excitability has not been investigated. Based on the observations in CA3 cells and the anti-convulsant properties of KOR agonists, we hypothesised that activation of KORs would decrease excitability of DG granule cells.

Using electrophysiological techniques, we found that activation of KORs with U6 actually increases the excitability of dentate gyrus granule cells. The boosting effect of U6 on the spiking behaviour of granule cells was prevented by use of the KOR antagonist BNI, or by inhibiting G-protein activation, and was also occluded by the A-type K^+ current blocker 4-AP. A surface crosslinking assay and subsequent protein analysis suggests that U6 affects the phosphorylation and trafficking of Kv4.2 subunits to the cell surface. With the emerging role of KORs in behaviours such as depression and epilepsy, it is becoming increasingly important to understand the impact and mechanisms of KOR activation, and these results add to this growing knowledge.

Methods

Electrophysiology

Subjects were 4- to 6-week-old 129SvE mice and all experiments were performed within the guidelines of the Tulane University Institutional Animal Care and Use Committee. Animals were anaesthetised using halothane and rapidly decapitated. Hippocampus slices (350 μm) were cut on a Vibratome. The cutting solution contained (in mM): 234 sucrose, 2.5 KCl, 1.25 NaH_2PO_4, 28 $NaHCO_3$, 7 $MgCl_2$, 0.5 $CaCl_2$, 7 dextrose, and 1 sodium ascorbate. Slices were incubated in a submerged chamber at 36°C for 30 min, and at room temperature thereafter until the time of recording. Individual neurons were visualised with an Olympus BX51WI fitted with differential interference contrast (DIC) optics using infrared illumination. Whole-cell patch recordings were made using the Axon MultiClamp 700B amplifier in current-clamp mode. The normal external recording solution contained (in mM): 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and dextrose 10, bubbled with 95% O_2–5% CO_2 at 35°C, pH 7.4. The internal pipette solution consisted of (in mM): 120 potassium gluconate, 20 KCl, 10 Hepes, 0.2 EGTA, 4 Mg_2ATP, 0.3 $Tris_2GTP$, 14 phosphocreatine, and 4 NaCl. Resting membrane potential (−65 mV) was not altered by any of the drug applications. Input resistance was

determined from the slope of a linear fit of the relationship between the change in membrane potential and the magnitude of the injected current. Access resistance was monitored throughout the experiment and any cell whose access resistance changed by more than 20% was excluded from analysis.

Drugs: U6, BNI, GDP-βS and 4-AP were purchased from Sigma (St Louis, MO, USA). A stock solution of U6 (1 mM) was made in 0.1 N HCl and it was added to the external solution on the day of experiment to a working concentration of 1 μM. The final concentration of HCl in the external solution was 0.1 μM. Application of 0.1 μM HCl alone did not affect any of the physiological measurements we used during these experiments (data not shown). A stock solution of BNI (1 mM) was dissolved in diH_2O and added to the external solution at a working concentration of 1 μM. Stock solutions of 4-AP (200 mM) and GDP-βS (50 mM) were dissolved in diH_2O and added to the internal solution at working concentrations of 5 mM and 1 mM, respectively, as indicated.

Surface crosslinking

Slices were prepared as above and the hippocampus dissected out. After cutting, hippocampus slices were given time to equilibrate before incubation with the agonist. Four to five slices were used per condition. Slices were incubated in the KOR agonist U65953 (1 μM) for 10 min. For crosslinking, slices were incubated in ice-cold aCSF containing 2 mM bis(sulfosuccinimidyl) suberate (BS3) for 30 min to label extracellular proteins. The reaction was quenched with 100 mM glycine. After washing the slices were placed in lysis buffer, sonicated and aliquots were taken for Western blot analysis. Proteins were separated by SDS-PAGE using an 8% gel and transferred to PVDF membrane. Membranes were blocked in TBS containing 0.1% Tween 20, 5% dried milk and 3% BSA. Four antibodies were used for the Western blots, as follows: the voltage-gated K^+ channel subunit 4.2 (Kv4.2) antibody (1:500, Chemicon), the phosphorylated Kv4.2 antibody (Anderson *et al.* 2000) (1:1000, generous gift from Dr Anne Anderson), the Mslo maxi-K^+ channel antibody (1:1000, NeuroMab) and the β-tubulin antibody (1:1000, Chemicon). The β-tubulin antibody was used as a loading control. All data represented have been normalized to β-tubulin. Imaging and quantification were performed using the Biorad Chemidoc XRS with Quantity One software.

Immunohistochemistry

Male 129/SvE mice (4–6 weeks) were anaesthetised and perfused intracardially with a saline rinse followed by 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Brains were removed and postfixed in 4% paraformaldehyde and 30% sucrose for 24 h. Coronal sections through the hippocampus were cut at 30 μm by a cryostat and collected in PBS. Sections were incubated for 24 h in the κ opioid receptor rabbit polyclonal antibody (Abcam, ab10566) diluted 1:3000 in PBS containing 0.1% Triton at 4°C. This antibody was previously used in the dorsal root ganglion of KOR knockout mice and only specific KOR recognition was observed (Tsai *et al.* 2010). Sections were stained using avidin–biotin complex immunocytochemistry with diaminobenzidine and hydrogen peroxide.

Statistics

Data were analysed using either repeated-measures analysis of variance (ANOVA), paired or unpaired Student's *t* tests, as relevant.

Results

While the KORs are highly expressed in the hippocampus, most of the studies to date have investigated KOR expression in rat and guinea pig. As this receptor displays species-specific distribution, we initially sought to confirm its presence in the hippocampus of mice, the subjects used in this study. As with other subjects, we found extensive staining in the granule cell layer and sporadic punctate staining in the hilus and molecular layer of the DG. There were also fibres stained in the molecular layer (Fig. 1). This verifies the presence of the KOR in the DG of mice.

We investigated the effects of activation of KOR on intrinsic membrane properties of DG granule cells. Counter to our expectations based on its reported anti-convulsant actions, application of 1 μM U6 for 20 min resulted in a significant increase in the number of action potentials elicited in response to 500 ms current injections at increasing depolarizations (two-way ANOVA with repeated measures; Interaction: $F_{(8,72)} = 3.3$; $P < 0.01$; U6 effect: $F_{(1,72)} = 156.3$; $P < 0.001$; Current injection effect: $F_{(8,72)} = 16.3$; $P < 0.001$). *Post hoc* Bonferroni testing revealed significant increases in number of spikes elicited in response to current injections ranging from 15 to 45 pA after exposure to U6 (15 pA, $P < 0.05$; 20–45 pA, $P < 0.001$; $n = 9$; Fig. 2*A* and *B*). Figure 2*A* shows a representative voltage trace in response to various current injections (−50, −20, 5, 45) before and 20 min after application of U6. This increase in spike number was not due to a change in cell input resistance (control *vs.* U6; 338.6 ± 20.0 MΩ *vs.* 354.5 ± 16.2 MΩ, $P = 0.54$, data not shown). The threshold for action potential generation was significantly lowered by U6 (control *vs.* U6; −44.9 ± 0.7 mV *vs.* −47.2 ± 0.6 mV, $P < 0.05$; Fig. 2*C*)

and the latency to first spike was significantly decreased by U6 (control *vs.* U6; 112.8 ± 10.0 ms *vs.* 85.0 ± 9.1 ms, $P < 0.01$ at the 45 pA depolarization; Fig. 2*D*). A change in the ISI in response to a 45 pA current injection was seen both at the beginning of a train of spikes (after 1st spike, control *vs.* U6; 63.7 ± 8.0 ms *vs.* 36.9 ± 6.1 ms, $P < 0.001$) and in the latter parts of the train (after 10th spike, control *vs.* U6; 69.9 ± 6.4 ms *vs.* 47.6 ± 6.2 ms; $P < 0.01$; Fig. 2*E*), suggesting an effect on spike frequency adaptation. In addition, U6 significantly enhanced the first spike half-width (control *vs.* U6; 1.3 ± 0.1 ms *vs.* 1.8 ± 0.1 ms, $P < 0.001$; Fig. 2*F* and *G*). U6 had no effect on membrane potential, suggesting that activation of KOR specifically affects voltage-gated currents. In summary, contrary to our expectation, the overall effect of U6 on individual granule cells was to increase membrane excitability.

While U6 is reported to be a selective KOR agonist, previous reports have suggested the possibility that U6 can have non-opioid actions (Alzheimer & ten Bruggencate, 1990). To confirm that U6 was acting at KORs, we tested the effects of BNI, a selective antagonist of the KOR. We preincubated slices in this antagonist 20 min before applying U6 to verify that the effects of U6 were attributable to KOR activation. As expected, a two-way repeated measures ANOVA suggested an effect of current injection on spike number ($F_{(8,54)} = 22.53$; $P < 0.001$; $n = 7$; Fig. 3*A* and *B*). A *post hoc* Bonferroni test, however, revealed no significant increases in number of spikes elicited in response to any of the current injections tested (5–45 pA) after exposure to U6 (Fig. 3*A* and *B*). As we saw with U6 alone, the input resistance was unaffected by application of U6, in the presence of BNI (BNI *vs.* U6 + BNI; 307.8 ± 10.1 MΩ *vs.* 303.2 ± 8.6 MΩ, $P = 0.41$). Even in the presence of BNI, U6 still reduced the threshold for spike initiation (BNI *vs.* U6 + BNI; -46.3 ± 1.5 mV *vs.* -49.3 ± 1.2 mV, $P < 0.01$; Fig. 3*C*) and decreased the ISI after the 1st spike (BNI *vs.* U6 + BNI; 67.9 ± 7.6 ms *vs.* 53.7 ± 9.5 ms, $P < 0.05$; Fig. 3*E*). Pre-exposure to BNI abolished the effect of U6 on the latency to first spike

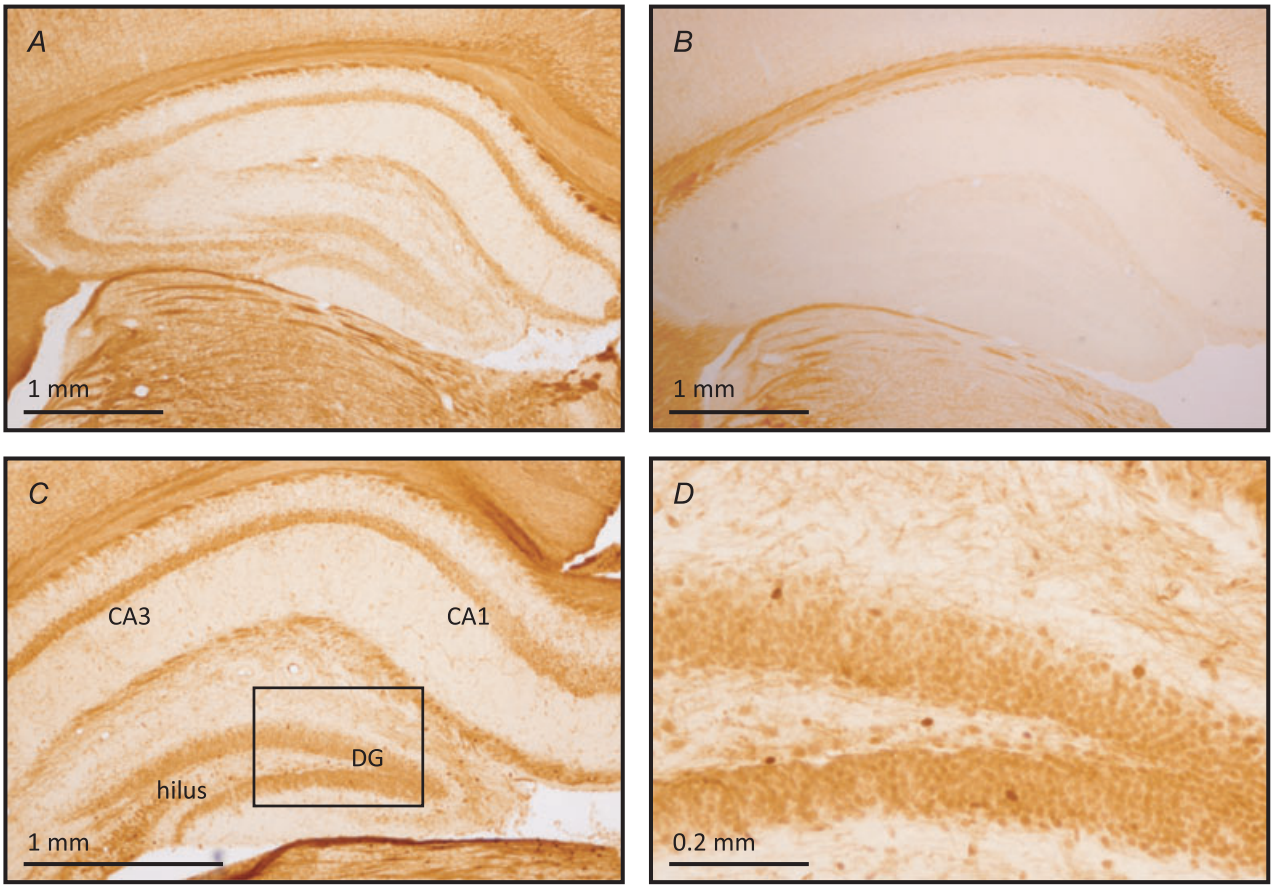

Figure 1. Immunoreactivity for the antibody against KOR is abundant in the dentate gyrus region of the hippocampus in mice
A, low power view of the hippocampus showing widespread KOR immunoreactivity. *B*, control section of hippocampus in which the primary antibody has been omitted. *C* and *D* are low and high power views, respectively, of KOR immunoreactive staining in the dentate gyrus. *D* is an enlarged area of the box shown in *C* showing the localisation of KOR immunoreactivity in dentate gyrus granule cells.

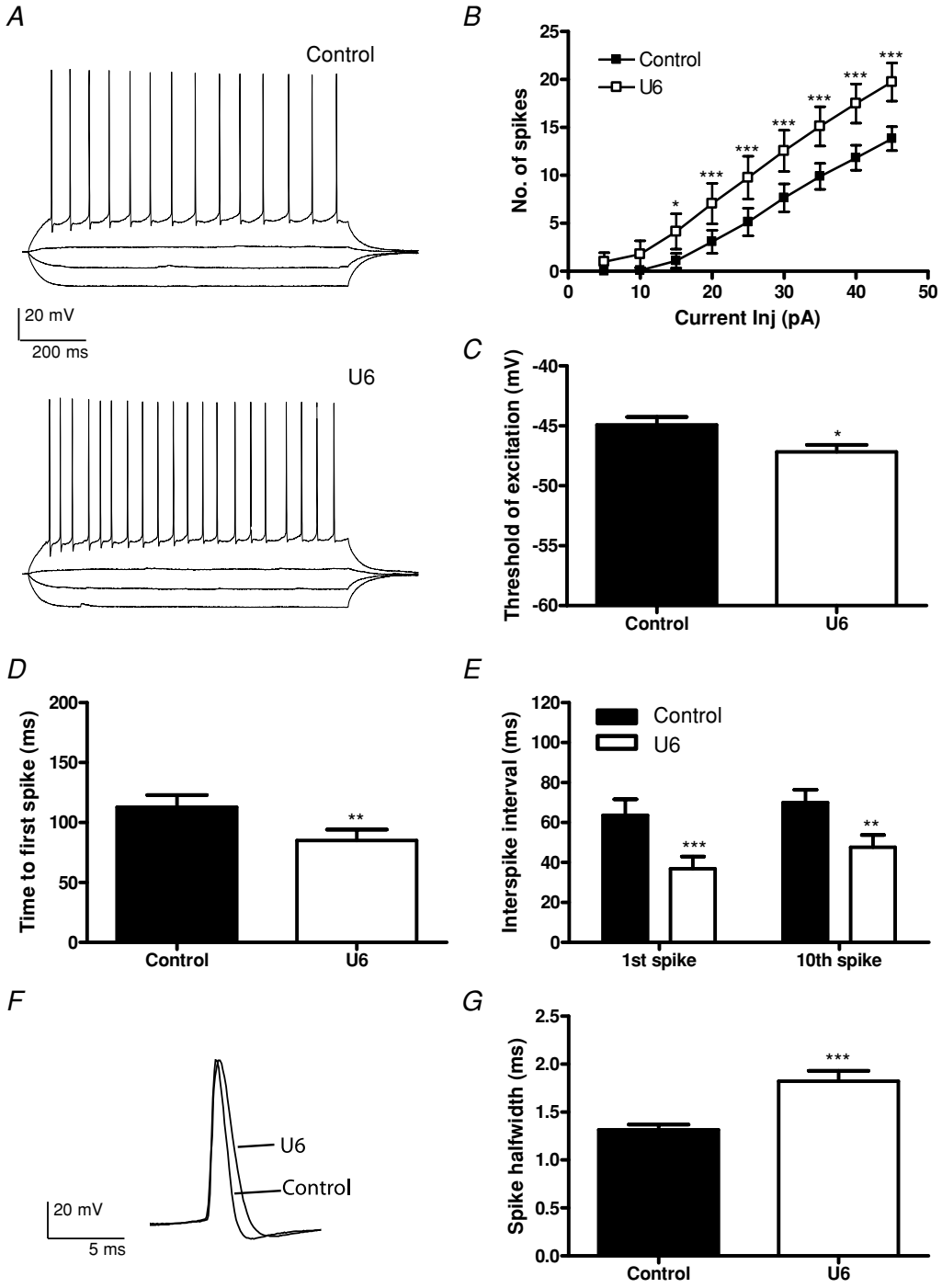

Figure 2. Activation of KORs increases the membrane excitability of dentate gyrus granule cells
A, representative voltage traces from DG granule cells recorded in the whole-cell patch-clamp configuration in response to current injections (−50, −20, 5 and 45 pA) before (upper) and after (lower) application of U6. *B*, graph representing the mean number of spikes evoked before and after U6 application for all cells tested ($n = 9$). *C*, bar graph representing the mean threshold for spike initiation in DG granule cells in response to a 45 pA current injection before and after U6 application. *D*, bar graph representing the mean latency to first spike in response to a 45 pA current injection before and after U6 application. *E*, mean ISI after the 1st and 10th spike in a spike train before and after U6 treatment. *F*, representative voltage traces showing action potential width before and after U6 treatment. *G*, bar graph showing mean spike half-width for the first spike in a train, after a 45 pA current injection, in the presence and absence of U6. All data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

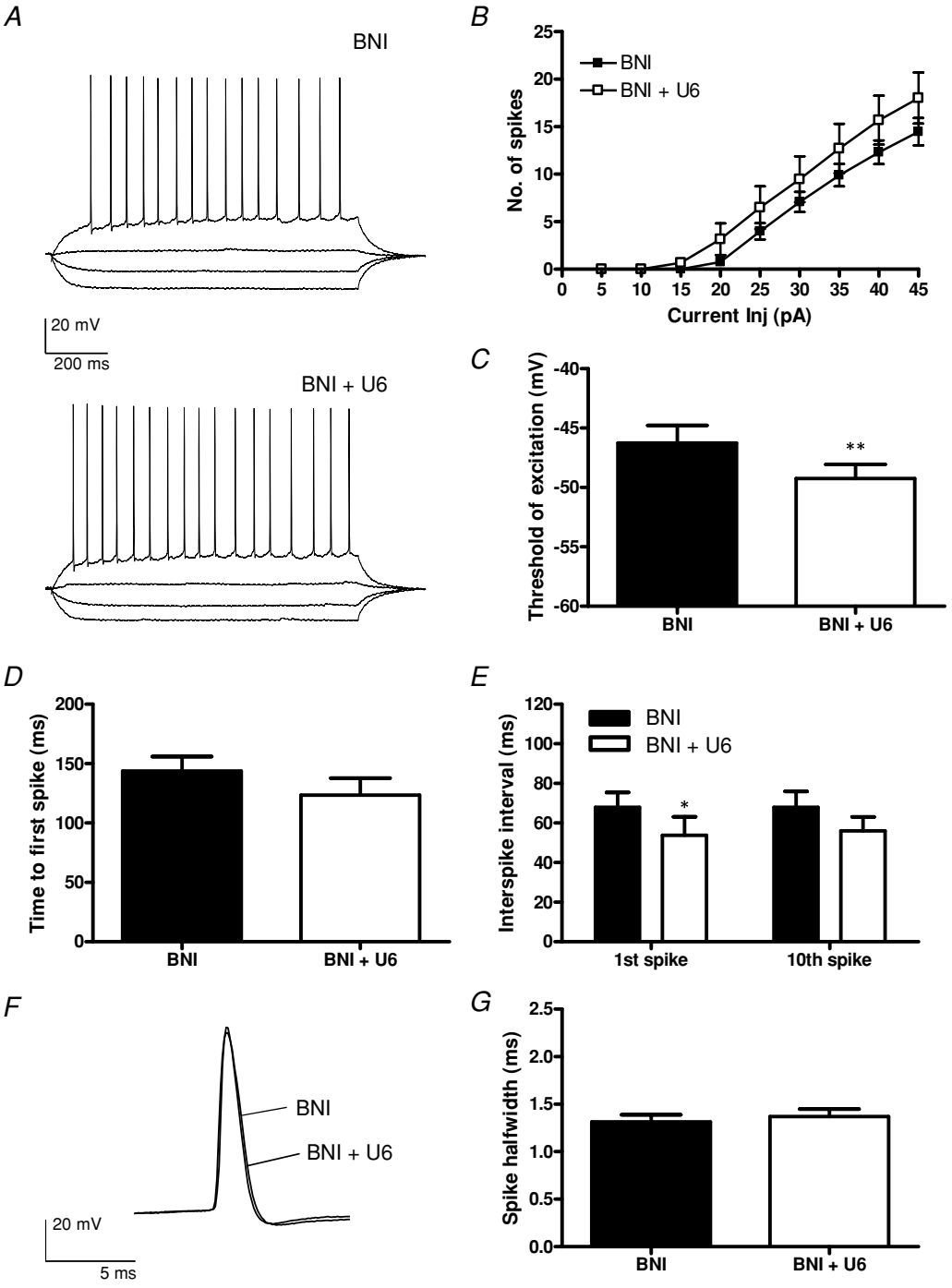

Figure 3. Preincubation of slices with the KOR antagonist BNI (20 min application) prevents the U6-induced increase in membrane excitability in dentate gyrus granule cells
A, representative voltage traces from DG granule cells recorded in the whole-cell patch-clamp configuration in response to current injections (−50, −20, 5 and 45 pA) before (upper) and after (lower) application of U6. *B*, graph representing the mean number of spikes evoked before and after U6 application for all cells tested ($n = 7$). *C*, bar graph representing the mean threshold for spike initiation in DG granule cells in response to a 45 pA current injection before and after U6 application. *D*, bar graph representing the mean latency to first spike in response to a 45 pA current injection before and after U6 application. *E*, mean ISI after the 1st and 10th spike in a spike train before and after U6 treatment. *F*, representative voltage trace showing action potential width before and after U6 treatment. *G*, bar graph showing mean spike half-width for the first spike in a train, after a 45 pA current injection, in the presence and absence of U6. All data are mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$.

(BNI *vs.* U6 + BNI; 143.8 ± 12.1 ms *vs.* 123.4 ± 14.4 ms, $P = 0.08$;Fig. 3*D*), and the ISI after the 10th spike (BNI *vs.* U6 + BNI; 68.0 ± 8.0 ms *vs.* 55.5 ± 7.0 ms, $P = 0.39$; Fig. 3*E*). Preincubation with BNI also antagonised the effect of U6 on the half-width of the first spike in a train (BNI *vs.* U6 + BNI; 1.3 ± 0.1 ms *vs.* 1.4 ± 0.1 ms, $P = 0.13$; Fig. 3*F* and *G*). The input resistance of cells that had been preincubated in BNI was about 10% lower than that of those from naive slices. This may account for the insignificant decrease in the number of spikes evoked at each depolarization by BNI preincubation alone. These results indicate that U6 may be acting through two distinct mechanisms (KOR and non-KOR) to enhance the excitability of dentate granule cells. The non-KOR-mediated effect lowers the spiking threshold and reduces the ISI directly after the initial spike in a train. Meanwhile U6 acting through the KOR affects the latency to first spike, spike width and ISI at later points in a train.

KORs are G-protein-coupled receptors. To confirm that the effects of U6 were mediated through G-protein activation, we added GDP-βS (1 mM) to the pipette solution. GDP-βS is a non-hydrolysable form of GDP. When added to the intracellular solution, GDP-βS prevents G-protein activation. GDP-βS alone did not change the membrane properties of granule cells. Furthermore, with GDP-βS in the pipette solution, U6 no longer had a significant effect on most of the physiological properties of dentate granule cells (Fig. 4). The average number of spikes evoked by a range of current injections was unchanged by the application of U6 while GDP-βS was in the pipette. A two-way repeated measures ANOVA revealed a significant effect of current injection on spike number ($F_{(8,54)} = 11.95$; $P < 0.001$; $n = 7$; Fig. 4*A* and *B*). *Post hoc* Bonferroni testing revealed no significant increase in number of spikes elicited in response to all current injections ranging from 5 to 45 pA after exposure to U6 (Fig. 4*A* and *B*). However, the input resistance was reduced slightly (GDP-βS *vs.* GDP-βS + U6; 348.8 ± 29.0 MΩ *vs.* 311.2 ± 30.0 MΩ; $P < 0.01$). The threshold for spike initiation was no longer affected by U6 (GDP-βS *vs.* GDP-βS + U6; −42.7 ± 1.1 mV *vs.* −45.2 ± 1.7 mV, $P = 0.06$;Fig. 4*C*). The latency to first spike was not affected at 125.7 ± 21.1 ms and 124.2 ± 35.6 ms, ($P = 0.94$) before and after U6 application, respectively (Fig. 4*D*). Also, in the presence of GDP-βS, U6 no longer reduced the ISIs at any point in the train of action potentials (after 1st spike, GDP-βS *vs.* GDP-βS + U6; 92.4 ± 17.9 ms *vs.* 78.5 ± 26.3 ms, $P = 0.59$; after 10th spike, GDP-βS *vs.* GDP-βS + U6; 79.3 ± 11.5 ms *vs.* 64.4 ± 10.1 ms, $P = 0.66$; Fig. 4*E*). Likewise, the spike half-width was unaltered (GDP-βS *vs.* GDP-βS + U6; 1.7 ± 0.1 ms *vs.* 2.0 ± 0.3 ms, $P = 0.16$; Fig. 4*F* and *G*). These results suggest that the effects of U6 were mediated by G-protein-coupled receptors.

As we did not see any change in cell input resistance or membrane potential, it is unlikely that U6 caused a change in resting membrane currents. The most likely explanation for the changes in intrinsic excitability is modulation of currents through voltage-gated channels. The reduced latency to first spike and increase in threshold and spike half-width are consistent with a decrease in the A-type potassium current (Kim *et al.* 2005). The A-current is a voltage-dependent, transient, outward current seen throughout the neurons of the CNS. It is rapidly activated with depolarization from rest, activates at subthreshold potentials and then rapidly inactivates, also in a voltage-dependent manner (Connor & Stevens, 1971).

A-current recordings in voltage clamp were difficult to obtain from granule cells as most of the current is out in the dendrites. In order to determine whether the effects of U6 on cell excitability are the result of changes in the A-type K^+ current we added 5 mM 4-AP, an A-type K^+ current blocker, to the intracellular solution. At this concentration 4-AP significantly reduces the A-type current (Jung *et al.* 2008). The cell was allowed to dialyse for 20 min before recording control traces after which U6 was applied for 20 min. Under these conditions U6 had no effect on the excitability of dentate gyrus granule cells. The number of spikes elicited in response to a series of current injections did not change after U6 application ($n = 5$; Fig. 5*A*). A two-way ANOVA with repeated measures revealed a significant current injection effect on spike number ($F_{(8,45)} = 6.4$; $P < 0.0001$; Fig. 5*A*). *Post hoc* Bonferroni testing confirmed that there was no significant change in cell spiking after U6 application. The presence of 4-AP did not influence U6's effect on input resistance which remained unaltered (4-AP *vs.* 4-AP + U6; 336.2 ± 25.5 MΩ *vs.* 318.5 ± 27.1 MΩ, $P = 0.65$). 4-AP blocked the effect of U6 on the spike threshold (4-AP *vs.* 4-AP + U6; −48.7 ± 1.2 mV *vs.* −51.0 ± 1.8 mV, $P = 0.08$; Fig. 5*B*), the latency to first spike (4-AP *vs.* 4-AP + U6; 81.3 ± 22.1 ms *vs.* 87.4 ± 33.5 ms, $P = 0.63$; Fig. 5*C*), ISI (1st spike, 4-AP *vs.* 4-AP + U6; 46.1 ± 23.5 ms *vs.* 57.2 ± 41.3 ms, $P = 0.58$; after 10th spike, 4-AP *vs.* 4-AP + U6; 59.2 ± 6.1 ms *vs.* 53.7 ± 10.3 ms, $P = 0.85$; Fig. 5*D*) and the spike half-width (4-AP *vs.* 4-AP + U6; 1.7 ± 0.1 ms *vs.* 1.8 ± 0.1 ms, $P = 0.08$; Fig. 5*E*). These results suggest that the effects of U6 on cell excitability are mediated through 4-AP-sensitive A-type currents.

In some areas of the brain, the A-type K^+ current is carried by the Kv4.x α subunits. In the DG, Kv4.2 and Kv4.3 are widely expressed and are believed to be responsible for the A-type current (Serodio & Rudy, 1998). We used an antibody directed against the Kv4.2 subunit to determine whether protein levels on the cell surface are altered by U6, which could underlie the physiological changes induced by U6. After the incubation period with U6, surface proteins were crosslinked and proteins were isolated and separated

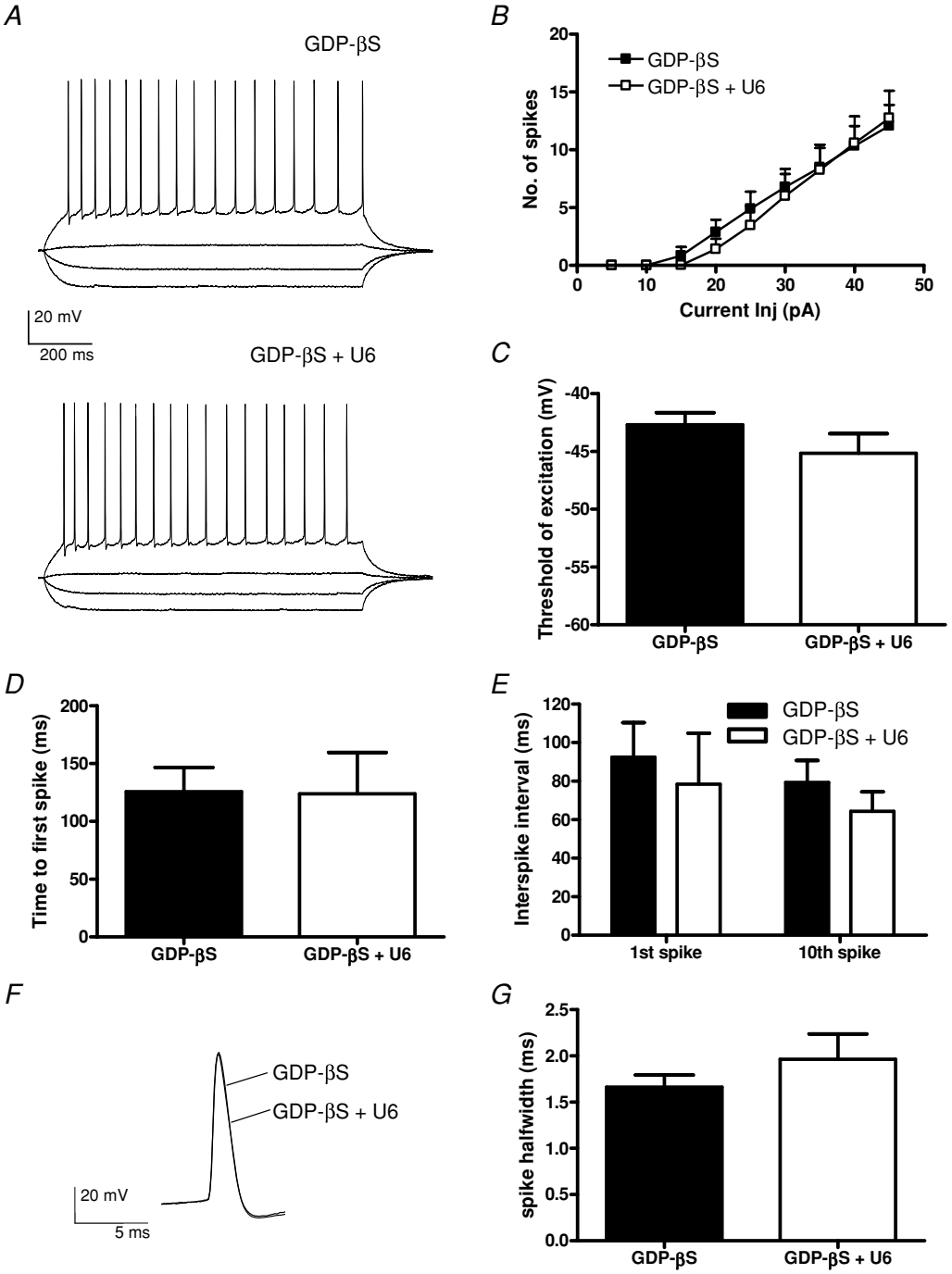

Figure 4. The effect of U6 is blocked by inclusion of GDP-βS, which blocks G-protein activation, in the intracellular solution

A, representative voltage traces from DG granule cells recorded in the whole-cell patch-clamp configuration in response to current injections (−50, −20, 5 and 45 pA) before (upper) and after (lower) application of U6. *B*, graph representing the mean number of spikes evoked before and after U6 application for all cells tested ($n = 7$). *C*, bar graph representing the mean threshold for spike initiation in DG granule cells in response to a 45 pA current injection before and after U6 application. *D*, bar graph representing the mean latency to first spike in response to a 45 pA current injection before and after U6 application. *E*, mean ISI after the 1st and 10th spike in a spike train before and after U6 treatment. *F*, representative voltage trace showing action potential width before and after U6 treatment. *G*, bar graph showing mean spike half-width for the first spike in a train, after a 45 pA current injection, in the presence and absence of U6. All data are mean ± SEM.

on SDS-PAGE gels. Crosslinked surface proteins are heavy and will remain at the top of a gel allowing them to be distinguished from intracellular proteins (Partis *et al.* 1983). The Kv4.2 antibody recognised a large amount of surface protein relative to intracellular protein (70 kDa), which suggests that most of the Kv4.2 protein is on the cell surface. Although the total amount of Kv4.2, surface and intracellular combined, did not change (Fig. 6*A* and *B*), the proportion of Kv4.2 on the cell surface was reduced after exposure to U6 ($n = 7$, $P < 0.05$). Thus, it appears that U6 reduced surface expression of the Kv4.2 subunits, perhaps by increasing its internalisation. In cultured hippocampus CA1 neurons, activity-dependent Kv4.2 internalisation is triggered by phosphorylation of serine (S)552 on the Kv4.2 subunit (Hammond *et al.* 2008). To determine whether U6-induced internalisation of Kv4.2 is triggered by phosphorylation at this site, an antibody specifically directed against the 552 phosphorylated form of this subunit was used (pKv4.2) (Anderson *et al.* 2000). This antibody did not recognise any crosslinked protein on the cell surface. This may be due to the crosslinking concealing the antibody recognition site or some other modification of the protein during the crosslinking process. In the intracellular fraction there was a marked increase in the pKv4.2 immunoreactivity after U6 incubation ($n = 5$, $P < 0.05$; Fig. 6*C*). These results suggest that KOR activation caused

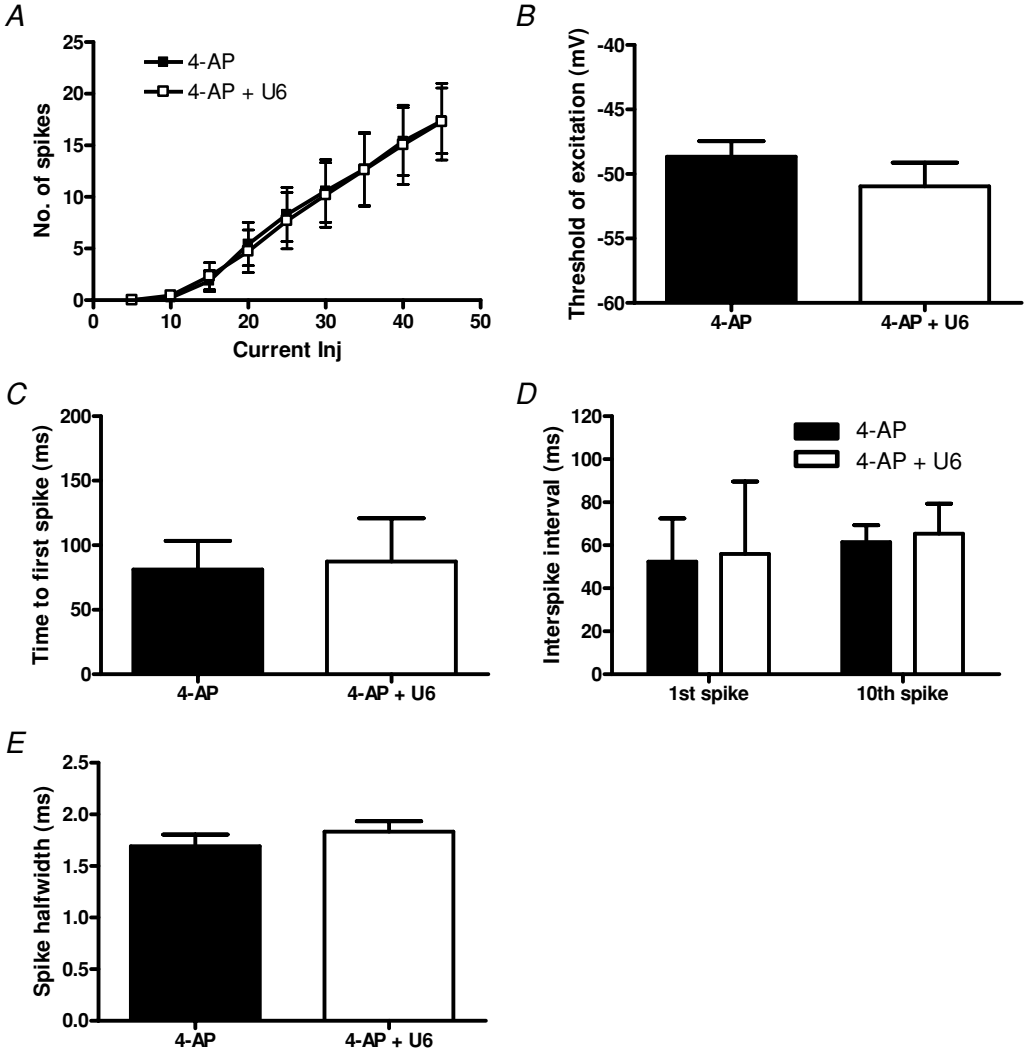

Figure 5. The A-type K^+ current blocker 4-AP occludes the effect of U6 on granule cell excitability
A, graph representing the mean number of spikes evoked before and after U6 application for all cells tested ($n = 5$). *B*, bar graph representing the mean threshold for spike initiation in DG granule cells in response to a 45 pA current injection before and after U6 application. *C*, bar graph representing the mean latency to first spike in response to a 45 pA current injection before and after U6 application. *D*, mean ISI after the 1st and 10th spike in a spike train before and after U6 treatment. *E*, bar graph showing mean spike half-width for the first spike in a train, after a 45 pA current injection, in the presence and absence of U6. All data are mean ± SEM.

a phosphorylation of S552 on the Kv4.2 subunit (Anderson *et al.* 2000; Schrader *et al.* 2002) and subsequently internalisation of the channel resulting in an increase in the excitability of the DG granule cell. In order to determine if the decrease in surface expression is an effect specific to Kv4.2, we investigated the BK channels as well. The BK channel is another surface membrane channel that may also participate in the changes in spiking that we observed. We used an antibody against the BK type mSlo K^+ channel to determine whether its protein expression

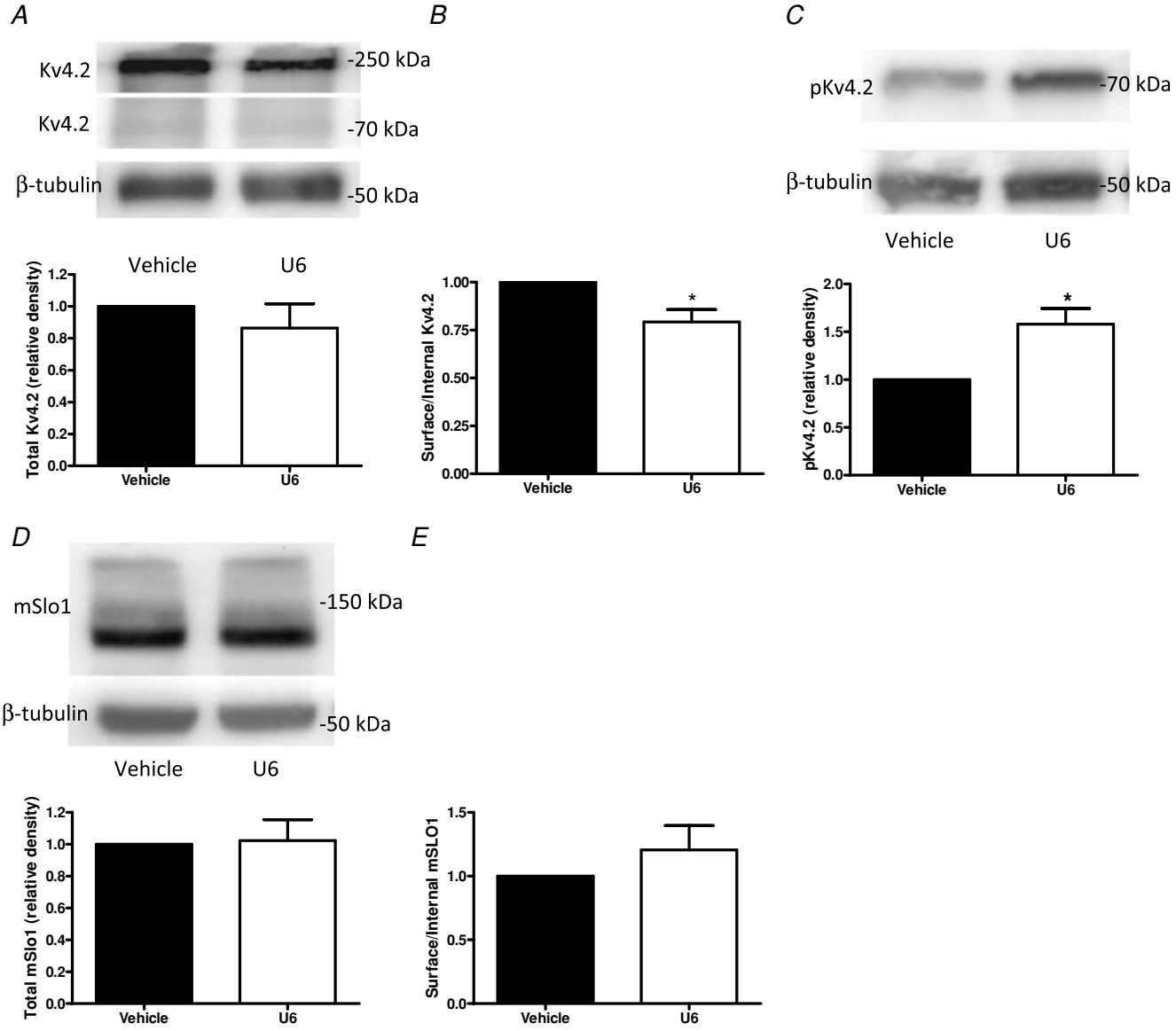

Figure 6. Surface expression of the Kv4.2 subunit is decreased by treatment of hippocampus slices with U6

A, upper panel: representative Western blots of hippocampus proteins showing immunoreactivity for the labelled proteins after incubation in U6 and crosslinking of surface proteins; lower panel: average total (surface and intracellular) immunoreactivity for Kv4.2 after exposure to U6 ($n = 7$). *B*, average surface/intracellular immunoreactivity for Kv4.2 after incubation with U6. *C*, upper panel: representative Western blots of hippocampus proteins showing immunoreactivity for the labelled proteins after incubation in U6 and crosslinking of surface proteins; lower panel: bar graph showing the average relative density of pKv4.2 immunoreactivity from the intracellular fraction of hippocampus extracts after incubation with U6 ($n = 5$). No surface protein was identified by the pKv4.2 antibody. *D*, the surface expression of the BK mSlo1 subunit is unaffected by treatment of hippocampus slices with U6. Upper panel: representative Western blot of hippocampus proteins showing immunoreactivity for the labelled proteins after incubation in U6 and crosslinking of surface proteins; lower panel: average total (surface and intracellular) immunoreactivity for mSlo1 after exposure to U6 ($n = 5$). *E*, average surface/intracellular immunoreactivity for mSlo1 after incubation with U6. Data are mean ± SEM and are normalized to vehicle conditions. $^*P < 0.05$.

is altered by U6. U6 had no effect on either the surface expression or the intracellular fraction of the mSlo1 subunit (Fig. 6*D* and *E*). This suggests that the effect on Kv4.2 surface expression is a specific effect.

Discussion

We determined the effects of κ-mediated opioid signalling on excitability of granule cells in the DG. Surprisingly, we found that activation of postsynaptic KORs enhanced the excitability of DG granule cells. The KORs appear to modulate the response of the cell to suprathreshold inputs and therefore have an important role in determining the output of the cell. Specifically, activation of KORs led to an increase in spiking activity in response to current injection. U6 increased the number of action potentials evoked at any given current injection, reduced the threshold of excitation, decreased the latency to first spike, increased the spike half-width and reduced the ISI throughout the spike train. The effects produced by U6 are antagonised by the presence of the non-hydrolysable GDP-βS in the patch pipette, confirming that these effects are mediated through G-proteins. Most of the effects were blocked by the κ-specific antagonist BNI, confirming that they are the result of activation of KORs, although U6 appears to have non-KOR-mediated actions as well. Finally, the effects of U6 were occluded by 4-AP, an A-type current blocker. Together these data suggest that KORs are located postsynaptically on DG cells and act to modulate voltage-dependent currents and granule cell excitability.

Much of the published data to date suggest mostly presynaptic actions of KORs. This study adds to mounting evidence suggesting that KORs also have postsynaptic actions (Moore *et al.* 1994; Madamba *et al.* 1999). Dynorphin A decreases excitability of CA1 cells from rat as measured via an enhancement of the M-current (Madamba *et al.* 1999). A decrease in excitability due to KOR activation is contrary to the results we have reported here. However, this dynorphin-mediated effect on I_M was not mimicked by U6. As U6 is only active at the κ_1 receptor, it is likely that the effect of dynorphin on I_M is mediated through κ_2 receptors. This is the predominant subtype found in rat (Zukin *et al.* 1988). The authors did not look at other membrane currents. Although there have been no previous reports on the excitatory effects of KOR agonists on the intrinsic properties of DG granule cells, both U6 and U50 directly increase cell excitability of CA3 pyramidal cells from guinea pigs (Alzheimer & ten Bruggencate, 1990). These compounds increased the input resistance and reduced the fast afterhyperpolarization following an action potential in these cells. Interestingly the κ_1 subtype is predominant in guinea pigs (Zukin *et al.* 1988). This effect, however, was mediated by non-opioid actions as it was not blocked by BNI or naloxone.

Mechanism of the effect

The regulation of several currents could play a role in the observed effects. We investigated the role of the A-type current using 4-AP applied in the pipette to specifically block K^+ currents in the postsynaptic cell. Application of 4-AP completely blocked the effects of U6. We investigated surface expression of the Kv4.2 channels that can underlie the A-type current. We observed effects on surface expression of Kv4.2 channels, but we did not see any effect of KOR activation on the protein expression of BK channels. This suggests that activation of KORs specifically modulates surface expression of Kv4.2. The lack of effect on surface expression of BK channels, however, does not preclude modulation of the kinetics of BK currents. Protein analysis confirmed that there is a loss of Kv4.2 from the cell surface after U6 exposure and an increase in phosphorylated Kv4.2 inside the cell.

The trafficking of Kv4.2 proteins, and consequently the A-type K^+ current, is extremely dynamic and very responsive to cell activity (Frick *et al.* 2004; Kim *et al.* 2007; Lei *et al.* 2008). The localisation and functional state of the Kv4 channel is highly regulated by the phosphorylation state of both the α (Kv4.x) and auxiliary subunits. Within the Kv4.2 subunit sequence phosphorylation at S552 regulates the biophysical properties of the channel (Schrader *et al.* 2002) as well as activity-dependent trafficking (Hammond *et al.* 2008). Consistent with these studies, we observed an increase in phosphorylation of Kv4.2 at S552, which was accompanied by a loss of Kv4.2 from the cell surface.

In the native cell, Kv4 subunits are associated with several different auxiliary subunits. One family of Kv4 interacting proteins are called the K^+ channel interacting proteins or KChIPs. Four subtypes of KChIPs have been found (KChIP1–4) which associate with different Kv4 subunits. KChIPs modulate the kinetic properties and localisation of Kv4 currents (An *et al.* 2000). Coexpression of KChIP3 with Kv4.2 in cultured cells endows the cell with more native-like A-type K^+ currents. It is also required for protein kinase A phosphorylation-dependent regulation of Kv4.2 (Schrader *et al.* 2002). KChIPs are also themselves substrates for modulation by various kinases. One site (S95), which is important for KChIP3 regulation of Kv4.2, is phosphorylated by G protein-coupled kinase 2 (GRK2). Recent evidence suggests that phosphorylation at this site disrupts KChIP3-mediated membrane expression of the Kv4 channel and results in smaller A-type K^+ currents (Ruiz-Gomez *et al.* 2007). GRKs are also known to be activated by KORs (Appleyard *et al.* 1999; Li *et al.* 1999; McLaughlin *et al.* 2003*b*). Therefore, direct kinase phosphorylation of the Kv4.2 subunit or the KChIP3 protein could be responsible for the surface localisation changes of Kv4.2 that we observed.

The physiological features of the A-type current, which activates rapidly and at sub-threshold potentials, in conjunction with its localisation in the cell, place it in a special position to have a large impact on the integration of inputs. Its colocalisation in the area of the cell that receives most of the excitatory inputs means that it is in a key position to modulate the strength of incoming signals. The striking gradient of A-type current along the dendritic shaft of pyramidal cells is responsible for the attenuation of back-propagating action potentials with distance from the soma (Hoffman *et al.* 1997). When the A-type current is reduced, somatically generated action potentials can propagate into the dendrites without attenuation and boost incoming signals. The A-type current is also an important determinant of spike width and accordingly, the duration of back-propagating action potentials is also increased when the A-type current is blocked (Hoffman *et al.* 1997; Kim *et al.* 2005). Back-propagating action potentials are important contributors to the induction of long-term potentiation (Magee & Johnston, 1997). The temporal pairing of subthreshold excitatory postsynaptic potentials with back-propagating action potentials is thought to be the key element in the induction of LTP in CA1 pyramidal cells. As modulators of back-propagating action potentials, it is not surprising that A-type currents also regulate the induction of LTP. Pharmacological blockade and gene deletion of Kv4.2 both enhance the amplitude of back-propagating action potentials and lower the threshold for LTP induction in CA1 pyramidal neurons (Ramakers & Storm, 2002; Chen *et al.* 2006). Subtle changes in A-type current activity, such as those resulting from kinase activation can still have profound effects on the amplitude of back-propagating action potentials (Hoffman & Johnston, 1998). Spike-timing-dependent plasticity is also regulated by the A-type current (Watanabe *et al.* 2002). Thus the postsynaptic effects of KORs can be powerful and influence the overall excitatory state of the cell as we have observed. As modulators of the A-type current, activation of KORs can ultimately control the impact of incoming signals, the strength of back-propagating action potentials and the induction of synaptic plasticity.

Functional implications

The hippocampus is the point of origin for the majority of epileptic activity seen during seizures of patients suffering from temporal lobe epilepsy (TLE) and, in some models of TLE, κ opioid agonists have anticonvulsant effects. The DG is a critical point for the entry of information into the hippocampus and as such κ opioid signalling here may serve an important regulatory role for the level of activity in the hippocampus. The excitatory effects of KOR activation presented here appear to conflict with the anti-convulsant effects reported for KOR agonists. The anti-convulsant effects of κ activation are probably mediated through the inhibitory effect of KOR activation on synaptic transmission (Wagner *et al.* 1992; Simmons *et al.* 1994; Terman *et al.* 1994; Salin *et al.* 1995). Presumably, the presynaptic effects of κ agonists are sufficient to override any pro-convulsive tendencies that result from the post-synaptic effects of KOR activation. Indeed, the excitatory postsynaptic effect of KOR activation may serve to ensure relevant signals are still conveyed effectively for example for memory formation, while the inhibitory presynaptic effect prevents aberrant signalling from being transmitted.

The roles of KORs in nervous system function are still being deciphered but it is becoming rapidly clear that KORs can have profound influences on brain and cellular activity. The data presented here reveal yet another locus for modulation of activity by KORs. As we continue to uncover the wide-ranging effects of KOR activation, we will hopefully reveal potentially new approaches for therapeutic interventions in a wide array of common diseases such as epilepsy and depression.

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Author contributions

The experiments were performed in the laboratory of L. A. Schrader. Both authors contributed to the conception and design of the experiments. The collection and analysis of the data was performed by C. M. McDermott. Both authors contributed to the interpretation of the data, and the drafting and revision of the manuscript.

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