available at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/brainres

BRAIN RESEARCH

Research Report

Factors that regulate KiSS1 gene expression in the hippocampus

*Amy C. Arai**, *Nathane Orwig*

Department of Pharmacology, Southern Illinois University School of Medicine, P.O. Box 19629, Springfield IL 62794-9629, USA

ARTICLE INFO

Article history:
Accepted 10 September 2008
Available online 19 September 2008

Keywords:
Kisspeptin
Metastin
GPR54
BDNF
Organotypic slice culture
Behavioral seizure

ABSTRACT

Kisspeptin is a C-terminally amidated peptide encoded by the KiSS1 gene. The peptide and its receptor GPR54 are abundant in the hypothalamus and have been implicated as gatekeepers for the onset of puberty and the development of the reproductive system. Interestingly, GPR54 is also highly expressed in granule cells of the hippocampal dentate gyrus, and in a previous study we showed that kisspeptin enhances excitatory synaptic transmission in these cells. The present study examined how expression of KiSS1 and GPR54 is regulated in rat hippocampus, using in vivo and in vitro preparations. In animals, a 3 h period of kainate-induced seizures significantly altered expression of both genes. KiSS1 mRNA showed a 3–4 fold increase which peaked 1–3 days post-seizure and subsided after one week. GPR54 mRNA, on the other hand, was reduced by 20–30% at 6–24 h. In organotypic hippocampal slice cultures, brief exposure to kainate produced a significant increase in KiSS1 mRNA with a time course comparable to that in vivo, and the effect was blocked by tetrodotoxin and CNQX. Chronic (7-day) treatment with picrotoxin, which induced a persistent four-fold increase in spike activity in multi-electrode recordings, caused a similar size but more persistent upregulation in KiSS1 mRNA. As in other studies, kainate and picrotoxin induced an upsurge in BDNF expression, but BDNF mRNA was also significantly increased when slice cultures were treated with kisspeptin. Taken together, KiSS1 expression is upregulated by neuronal activity and activation of GPR54 by kisspeptin may in turn contribute to sustain basal BDNF levels required for hippocampal function. In additional experiments, KiSS1 mRNA was found to be increased after orchidectomy and thus expression may be regulated also by gonadal hormones.

1. Introduction

The KiSS1 gene was originally identified in melanomas as conferring suppression of metastasis (Lee et al., 1996), and a peptide encoded by this gene was subsequently shown to influence migration and other cellular processes in a wide range of cells (Lee and Welch, 1997; Masui et al., 2004). The primary translation product of the KiSS1 gene is processed proteolytically to yield a 54-amino acid carboxy-terminally amidated sequence that appears to be the primary active peptide and is variously named metastin, kisspeptin, or kisspeptin-54. However, shorter fragments containing the same C-terminus, but only 14, 13, or 10 amino acids, have been detected in tissues and found to be active with equal or

* *Corresponding author*. Fax: +1 217 545 0145.
E-mail address: aarai@siumed.edu (A.C. Arai).

0006-8993/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.brainres.2008.09.031

higher affinity than the parent peptide (Kotani et al., 2001; Ohtaki et al., 2001).

In 2001, several laboratories (Ohtaki et al., 2001; Muir et al., 2001; Kotani et al., 2001) discovered that the effects of these peptides are mediated by a G-protein coupled receptor that had been named GPR54 in rats (Lee et al., 1999). Activation of GPR54 in most cell lines appears to couple to $G\alpha_{q/11}$ and increase cytosolic calcium via stimulation of phospholipase C and phosphatidylinositol turnover (Kotani et al., 2001; Muir et al., 2001; Brailoiu et al., 2005). GPR54 is expressed in a wide range of tissues that include the brain, placenta, liver, and pancreas. In the brain, GPR54 was found to be particularly abundant in regions of the hypothalamus that regulate reproduction, and subsequent studies led to the discovery that the kisspeptin/GPR54 system plays a central role in controlling release of gonadotropin releasing hormone (GnRH) from hypothalamic neurons (Seminara et al., 2003; Funes et al., 2003; Gottsch et al., 2004; Navarro et al., 2004; Han et al., 2005). The importance of this system for the maturation of the gonadal organs has been illustrated most vividly by the discovery that some forms of hypogonadism are the result of mutations in GPR54 that render the receptor inactive (de Roux et al., 2003; Seminara et al., 2003; Semple et al., 2005). Kisspeptin receptors on GnRH-releasing neurons are therefore now considered a key factor for controlling sexual maturation and estrous cycles. Moreover, given that kisspeptin is also present in ovaries (Castellano et al., 2006) and that GPR54 controls trophoblast insertion into the uterine wall during pregnancy (Bilban et al., 2004), it is now widely thought that controlling reproduction is the primary role of this peptide system.

GPR54 is expressed at high density also in specific brain regions outside the hypothalamus, such as in the amygdala and in the hippocampal dentate gyrus (Lee et al., 1999), but there is as yet little information about the function of this peptide system in these areas. In a recent study we have shown that activation of GPR54 in dentate gyrus granule cells produces a dramatic increase in the amplitude of excitatory synaptic responses (Arai et al., 2005). The effect was abolished by the G-protein inhibitor GDP-β-S and the calcium chelator BAPTA, as well as by inhibitors of MAP kinases, calcium-calmodulin dependent kinase II (CaMK II) and tyrosine kinases. Analysis of miniature events indicated that kisspeptin increased event amplitude, but not event frequency, indicating that it acted post-synaptically. Kisspeptin did not affect membrane properties, and thus its mode of action in the dentate gyrus appears to be different from that in hypothalamic neurons where kisspeptin causes depolarization and enhanced spiking even when excitatory synaptic transmission is blocked (Han et al., 2005; Pielecka-Fortuna et al., 2008).

In our previous study we also showed by using RT-PCR that mRNA for KiSS1 is present in the dentate gyrus (Arai et al., 2005). The purpose of the present study has been to examine how expression of receptor and peptide is regulated. In the hypothalamus, KiSS1 expression is controlled by feedback from gonadal hormones (Smith et al., 2005). However, the most common factor of gene regulation in the brain is the level of neuronal activity. To test this, limbic seizures were induced by peripheral injection of kainate, using an experimental paradigm that has been widely employed to examine how neuronal activity influences gene expression in the hippocampus (Gall, 1988, 1993; Sperk, 1994). Parallel experiments were conducted with organotypic hippocampal slice cultures in which activity was enhanced by applying kainate or by blocking GABAergic inhibition with picrotoxin. Both in vivo and in vitro studies suggest that KiSS1 mRNA is substantially upregulated by neuronal activity.

2. Results

2.1. *Comparison of KiSS1 mRNA content across brain regions*

Kisspeptin immunoreactivity has been reported to be low or undetectable in cortex and hippocampus (Brailoiu et al., 2005), but we have previously shown that the hippocampus does express KiSS1 mRNA (Arai et al., 2005). To obtain an estimate for regional differences in the density of KiSS1 expression, we compared mRNA in the hippocampus with that of the hypothalamus and amygdala, using semi-quantitative RT-PCR. As shown in Fig. 1A, the hippocampus was found to contain about 50 to 100 times lower KiSS1 mRNA than the other two regions, which may explain in part why earlier studies did not detect the peptide. Expression in the hippocampus changed minimally during development, increasing at most two-fold between PND1 and PND10 and then remaining at the same level until PND50 (not shown).

2.2. *Kainate-induced seizures in vivo*

Peripheral injection of kainate is an experimental paradigm that has often been used as a model for temporal lobe epilepsy (Nadler, 1981; Sperk, 1994). Within 2 h of kainate injection, animals exhibit a spectrum of behavioral seizures ranging from wetdog shake to more advanced seizure stages that include forelimb clonus, rearing/falling and clonic–tonic convulsions. A few hours of seizure activity was previously described as being sufficient to increase expression of immediate early genes, including BDNF, and neuropeptides such as enkephalin and Neuropeptide Y (Gall 1988, 1993). We therefore used this paradigm to test if expression of mRNA for kisspeptin and GPR54 is modulated by these treatments.

Fig. 1B shows changes in KiSS1 mRNA after kainate treatment in the hippocampus, amygdala and hypothalamus, three brain regions that are of particular interest because of their high expression of GPR54. Seizure activity was observed for 3 h and then suppressed by injection of phenobarbital. The brains were harvested 21 h later and the samples were subjected to semi-quantitative RT-PCR. All the signals were corrected relative to the content of actin mRNA. An approximately three-fold increase in KiSS1 mRNA was observed in the hippocampus in those animals which exhibited major convulsions (average seizure score = 4), while animals that exhibited no or only low-grade seizures (e.g. wetdog shake) were not different (102 ± 14%, $n = 10$) from saline-treated animals ($n = 15$). To examine the time course of KiSS1 expression in the hippocampus, the brains of 23 animals exhibiting high-grade seizures were harvested 2–3 h, 6 h, 1 day, 3 days and 1 week

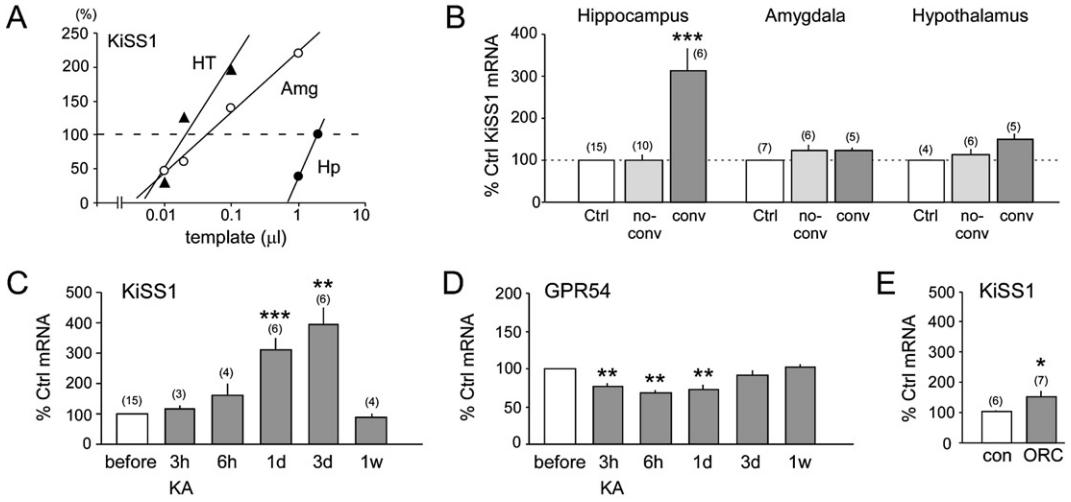

Fig. 1 – Expression of KiSS1 and GPR54 after post-kainate seizure. (A) Relative content of KiSS1 mRNA in the hippocampus (Hp), amygdala (Amg) and hypothalamus (HT); representative data. A total of 1 µg of RNA was submitted to RT reaction, and PCR was performed with 0.01–2 µl of the DNA template, as indicated on the X-axis. Optical density of the PCR product (202 bp) run on a polyacrylamide gel was normalized to that of the hippocampus (2 µl DNA template). (B) Effect of behavioral seizures on KiSS1 mRNA expression. Sprague–Dawley rats were injected with kainate (KA, 8–12 mg/kg, i.p.) and behavioral seizures were evaluated using a modified Racine scale. After 3 h, seizures were suppressed with phenobarbital (60 mg/kg, i.p.). Brain samples were collected 24 h after KA injection. Columns show the mean and s.e.m. of KiSS1 mRNA relative to that of saline-injected rats. The number in parenthesis indicates the number of animals. 'Conv' denotes the group of animals that exhibited convulsions higher than score 4, while 'No-conv' corresponds to a group of animals that showed score 0 or 1 (wetdog shake) during the 3 h period. ***, $p<0.001$ (vs control and vs no-convulsion). (C, D) Time course of changes in mRNA in animals which exhibited convulsions higher than score 4. The samples were collected at the indicated time points after KA injection. **, $p<0.01$; ***, $p<0.001$. (E) Level of KiSS1 mRNA in the hippocampus of male rats subjected to orchidectomy (ORC), normalized to that of a control group of sham-operated animals; the data are plotted using the same Y-axis scale as in C. The animals were sacrificed 3 weeks after surgery. *, $p<0.05$.

after kainate treatment. As shown in Fig. 1C, a modest increase (to 160±34% of control, $n=4$) was observed already after 6 h, but peak changes (3–4 fold) were found 24 and 72 h after kainate application. After one week, mRNA levels had returned to baseline. Phenobarbital treatment alone had no significant effects (88–114% of control) on KiSS1 and GPR54 expression when compared with saline-treated control animals. Very different results were obtained for GPR54 mRNA which was *decreased* after the seizure. Also, the reduction was largest at the 6 and 24 h time points (67±4% of saline control at 6 h, $n=4$, $p<0.001$) and expression returned to baseline already after 3 days. Overall, however, the change in GPR54 mRNA was much less pronounced than that for KiSS1. In the hypothalamus and amygdala, changes in KiSS1 mRNA were comparatively minor and not significantly different between animals with and without seizures (Fig. 1B).

In the hypothalamus, gonadectomy leads to major changes in KiSS1 expression which can be prevented by estrogen or testosterone replacement (Navarro et al., 2004; Smith et al., 2005), but regulation appears to be complex as KiSS1 expression was increased in some subregions and reduced in others. To examine whether expression in the hippocampus is also influenced by gonadal hormones, we measured mRNA three weeks after removing the testes according to the protocol of Navarro et al. (2004). A significant increase in KiSS1 mRNA was observed compared to sham-operated animals (150±19% of control, $p<0.05$, $n=7$; Fig. 1E), but the change was not nearly as large as that reported for the hypothalamus (3–4 fold) or the increase seen after kainate-induced seizures. No changes were detected in GPR54 expression (not shown).

2.3. *Kainate-induced seizures in vitro*

Organotypic hippocampal slice cultures were used to test whether kainate treatment under more controlled experimental conditions causes similar changes in gene expression. This preparation was previously shown to exhibit robust increases in the expression of immediate early genes and morphological changes after treatment with convulsants (Routbort et al., 1999). Sets of 12–16 slices were placed on Millicell culture plate inserts and maintained in culture until they had evenly thinned out (14–28 days; del Cerro et al., 1994). They were then exposed to 7 µM kainate for 6 h and harvested immediately afterwards or after 1, 3 and 7 days. To assess effects on neuronal activity, some cultures were transferred to a special culture dish with an 8×8 electrode array (Shimono et al., 2000; Kessler et al., 2008) and maintained on this array for one week to measure neuronal firing during and after kainate exposure. As shown in Fig. 2A, kainate treatment caused a large increase in spontaneous spike activity (5.7-fold) within 1 min of application which was then followed by a nearly total cessation of spike activity in the interval between 5 min and 6 h, presumably due to a sustained depolarization (Fig. 2B). Spike activity reappeared 1 day after washing out kainate and gradually returned to baseline levels.

KiSS1 mRNA showed changes in expression with a time course similar to that seen in vivo. mRNA remained unchanged after 6 h, was increased after 1–3 days and returned to baseline after 7 days (Fig. 2C). The increase was not as large as in vivo but statistically significant (144±16% of control at 3 days, $p<0.05$, $n=6$). The mRNA for GPR54 did not show measurable changes in these tests. The increase in KiSS1 mRNA was blocked by tetrodotoxin plus CNQX (Fig. 2D) and thus depends on activation of AMPA and kainate receptors and neuronal activity. In accordance with previous studies using cultured slices (Poulsen et al., 2004), mRNA for the neurotrophic factor BDNF was robustly increased (240±35%, $p<0.01$, $n=9$) after kainate treatment. This increase was largest at the 6 h timepoint and returned to baseline after 3 days.

2.4. *Effects of a chronic increase in excitability in vitro*

The kainate treatment we employed produced intense depolarization, but only for a limited time (6 h exposure). In further experiments we examined if expression of KiSS1 is enhanced also if neuronal activity is increased in a more graded but sustained manner. Following the procedure of Koyama et al. (2004), slice cultures were exposed for one week to 50 μM picrotoxin in order to block $GABA_A$ receptors. Figs. 3A and B illustrate that this treatment produced a 3–4 fold increase in the mean number of spikes that persisted over the entire test period. In CA1, this increase remained constant over the entire week, while activity in the dentate gyrus appeared to increase progressively from 2.3-fold initially to 8.8-fold at the end of this period. As shown in Fig. 3C, mRNA for KiSS1 was increased to 161±17% of control at the end of this test period ($p<0.01$, $n=7$), and thus to an extent comparable to that measured 1–3 days after kainate exposure. mRNA for GPR54 did not change significantly after picrotoxin treatment. BDNF expression in these tests was increased to 337±59% ($p<0.05$, $n=7$), which is again similar to that after kainate treatment (Fig. 3D, right).

2.5. *Kisspeptin enhances BDNF expression*

Expression of the trophic factor BDNF is enhanced by neuronal activity through mechanisms involving an increase in intracellular calcium (Poulsen et al., 2004). Since effects of

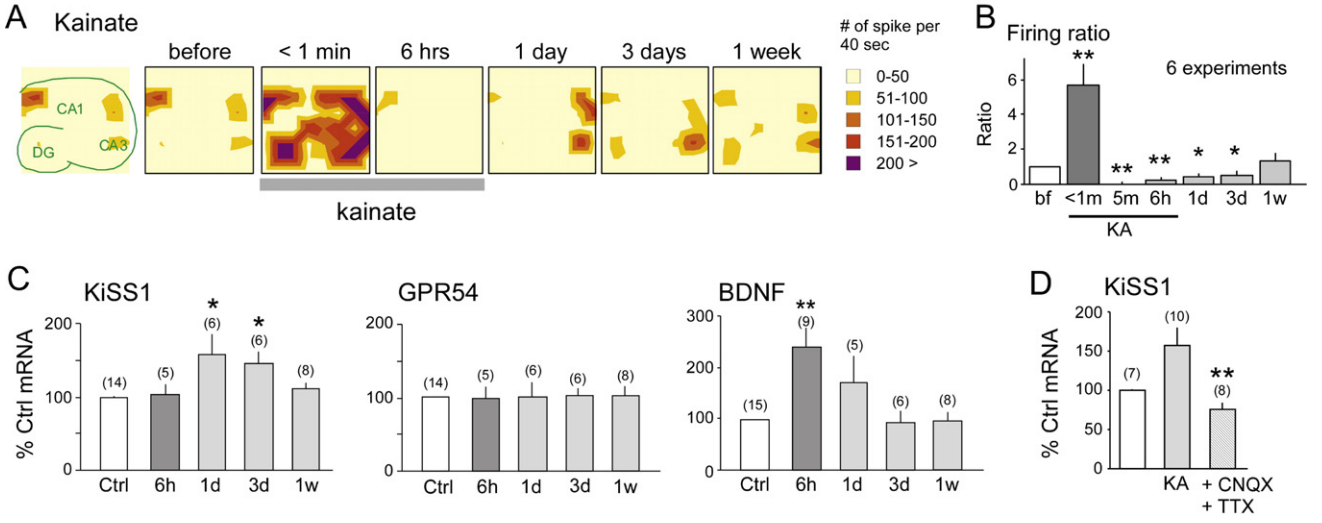

Fig. 2 – Expression of KiSS1 and GPR54 in organotypic hippocampal cultures after kainate treatment. Organotypic hippocampal slice cultures prepared from PND 10–12 animals were maintained for 14–28 days on a methylcellulose membrane at the interface between atmosphere and medium. The slices were then used for electrophysiology and gene expression experiments. (A) Slice cultures were transferred to a recording dish with an 8×8 microelectrode array, and spontaneous firing was monitored daily until activity had stabilized. Activity was counted as a spike when the potential exceeded 2–3 times the SD of the baseline potential. Recording was typically conducted in 40 s sweeps. The total number of spikes at each electrode was binned in increments of 50 events and plotted as spatial contour graph. Left: Baseline activity across hippocampal subregions. The activity contour graph was superimposed with a slice image in which subregions are identified. Right: Representative experiment for the changes in neuronal activity during and after kainate treatment. The slice was exposed to 7 μM kainate for 6 h (indicated with a horizontal bar). The first recording was taken immediately after addition of kainate. After washout of kainate, neuronal firing was monitored for 1, 3 and 7 days. (B) Group data for the time course of total spike activity. The number of spikes at all 64 electrodes was summed and normalized to that before (bf) addition of kainate ("Firing ratio"). Each column represents the mean and s.e.m. of 6 experiments. *, $p<0.05$; **, $p<0.01$, paired *t*-test. (C) Time course of changes in KiSS1, GPR54 and BDNF expression. Pools of 3–6 slices were exposed to kainate (7 μM) or control medium for 6 h, then rinsed and put back into regular medium. They were harvested at the end of kainate treatment (6 h), and 1 day, 3 days and 1 week after treatment. Semi-quantitative RT-PCR was conducted and the content of KiSS1, GPR54 and BDNF mRNA was normalized to that of the control slices. Columns represent the mean and s.e.m. of the number of experiments indicated in parenthesis. (D) Effect of blocking glutamatergic transmission and neuronal discharges on KiSS1 gene expression. Slices were treated with 7 μM kainate for 6 h in the presence and absence of 20 μM CNQX plus 2 μM tetrodotoxin (TTX) and harvested 18 h later. **, $p<0.01$ vs KA-treated group.

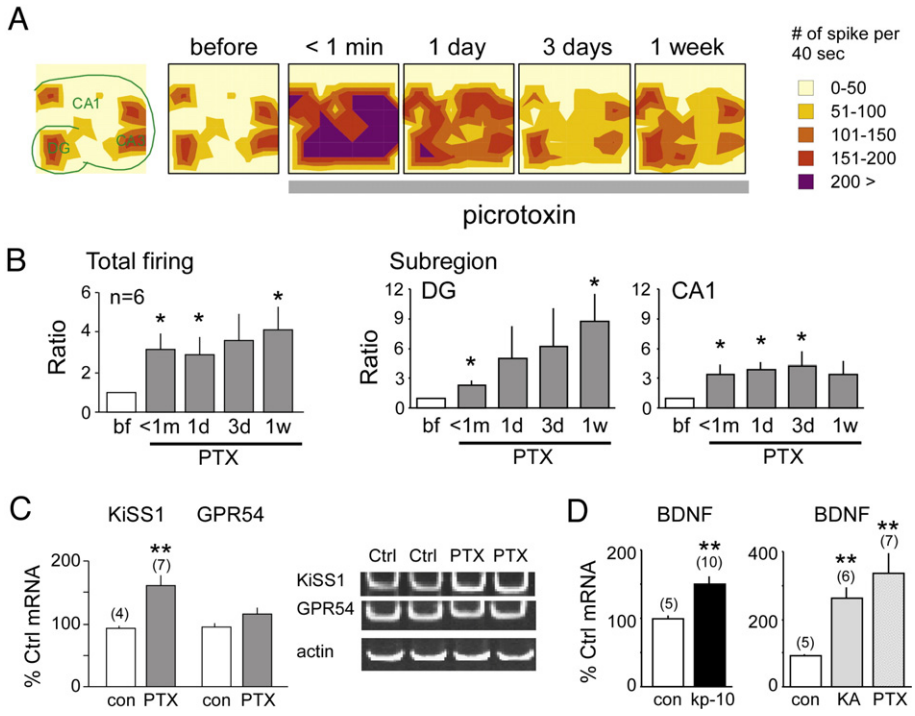

Fig. 3 – Expression of KiSS1 and GPR54 in organotypic hippocampal cultures after 1 week of sustained hyperexcitability. Organotypic hippocampal slice cultures were continuously exposed to 50 μM picrotoxin (PTX) for 1 week and then harvested for semi-quantitative RT-PCR. (A) Representative experiment showing spike activity before and during treatment with picrotoxin. The total number of spikes during a 40 s recording period was binned at 50 events and plotted as spatial contour graph. (B) Group data for the changes in firing rate. 'Ratio' denotes the number of spike events relative to that before picrotoxin (6 experiments). Left: ratio of spike number at all electrodes. Right: Ratio of spike number in dentate gyrus and CA1. *$p<0.05$, paired t-test, 1-tailed. (C) Expression of KiSS1 and GPR54 mRNA after one week of chronic treatment with picrotoxin, normalized to that of slices kept in control medium. Each column represents the mean and s.e.m. of the number of experiments shown in parenthesis. **, $p<0.01$. (D) Regulation of BDNF expression by kisspeptin and other agents that increase neuronal activity. Left: Organotypic slice cultures were exposed to 10 μM kisspeptin-10 (kp-10) for 6 h and then harvested to measure BNDF mRNA. Right: BDNF expression after 6 h of kainate treatment and after 1 week of picrotoxin treatment. **, $p<0.01$ vs the corresponding control group.

kisspeptin on synaptic responses in the dentate gyrus also require post-synaptic calcium (Arai et al., 2005), we studied further the possibility that exposure to kisspeptin enhances basal BDNF expression. To test this, mRNA for BDNF was measured after exposing organotypic hippocampal slice cultures for 6 h to 10 μM of the decapeptide kisspeptin-10. As shown in Fig. 3D, kisspeptin caused a statistically significant increase in BDNF mRNA to 149±12% ($p<0.01$, $n=10$).

3. Discussion

The main finding of this study has been that expression of kisspeptin is strongly regulated by neuronal activity. By far the largest changes in kisspeptin expression were obtained after limbic seizures induced with kainate. However, significant increases were also observed when kainate was briefly applied to hippocampal cultures and when neuronal activity was increased in a sustained manner with picrotoxin. The time course of the response to kainate was similar in vivo and in vitro, with mRNA reaching a peak level at 1–3 days and then returning to baseline. This time course is similar to that for many neuropeptide genes activated by convulsants (Gall, 1993) and it differed clearly from that for BDNF mRNA which reaches a peak after 3–6 h according to published studies (Poulsen et al., 2004) and in our experiments. The finding that kainate and picrotoxin treatment produced similar size changes in KiSS1 mRNA indicates that the enhancement of expression was caused by increased neuronal activity rather than activation of specific receptors. The picrotoxin experiments further showed that upregulation of KiSS1 expression can be sustained over prolonged periods of neuronal hyperactivity. No overt signs of cellular degeneration were observed after any of these treatments (not shown), but it can presently not be ruled out that latent neurodegenerative changes initiated by these treatments contributed to the enhanced KiSS1 expression. Whether upregulation of KiSS1 is observed after more physiological enhancement of neuronal activity remains to be examined in the future. In the amygdala and hypothalamus, seizure activity had only minor effects. This could indicate that KiSS1 expression is regulated differently in these regions or it may simply reflect the fact that AMPA and kainate receptors, the targets of kainate, are particularly abundant in the hippocampus (Monaghan et al., 1984).

A further potentially significant observation has been that expression of BDNF was enhanced by exogenous application of kisspeptin. BDNF is of central importance for dentate gyrus function. It is present in high concentration in the mossy fiber axons of the dentate granule cells and in the inner molecular layer (Conner et al., 1997), and among its many actions are to upregulate synaptic transmission at perforant path synapses and to promote synaptic plasticity (Messaoudi et al., 1998). BDNF expression often goes parallel with neuronal activity: a massive increase in BDNF mRNA is observed shortly after seizure onset (~6 h, Isackson et al., 1991), but moderate and persistent BDNF protein expression (2–3 fold) was noted even after 10 days as far as neuronal activity remained high (Koyama et al., 2004). For these and other reasons, BDNF has been implicated as a major factor underlying the seizure-induced sprouting of mossy fibers into the inner molecular layer (Koyama et al., 2004). As in previous studies, BDNF mRNA was increased several-fold when hippocampal cultures were exposed to kainate and picrotoxin. In addition, however, we found that kisspeptin application alone was also able to increase basal BDNF expression. This indicates that kisspeptin could induce longer lasting changes in neuronal activity via enhanced expression of BDNF, in addition to its immediate effects on synaptic transmission. It should be noted that kisspeptin in general did not change the resting membrane potential of DG neurons (Arai et al., 2005). Thus, the increase in BDNF expression is presumably the result of an activation of intracellular signaling cascades rather than a consequence of altered neuronal activity.

When the findings from this study are combined with those from our previous report (Arai et al., 2005), the interesting possibility emerges that the kisspeptin-GPR54 system forms a positive feedback loop in which granule cell excitability is enhanced by release of kisspeptin, which in turn would upregulate expression of kisspeptin. Moreover, enhancement of basal BDNF expression by kisspeptin and subsequent potentiation of synaptic responses by BDNF could constitute a second level of amplification. Positive feedback of this kind is normally held in check by strong inhibition (Coulter and Carlson, 2007), but it could become significant in pathological situations such as in temporal lobe epilepsy in which one often finds a loss of inhibitory neurons and an enhancement of excitatory feedback as a result of mossy fiber sprouting (Scharfman, 2002). However, before exploring these possibilities further, it will be important to resolve a number of more elementary issues such as whether upregulation of gene expression leads to a comparable increase in the release of kisspeptin, whether endogenously generated kisspeptin reaches concentrations sufficient for effectively activating GPR54, and whether or not prolonged exposure to kisspeptin causes significant downregulation of the receptor. It also remains to be studied whether all or only a subset of cells in the dentate gyrus generate kisspeptin and whether the peptide is similarly effective across synapses formed by different granule cell afferents.

In the hypothalamus, feedback from circulating gonadal hormones appears to be a major factor for regulation of KiSS1 expression and in one study it has been shown that the estrogen receptor ERα can associate with the transcription factor SP1 to regulate KiSS1 expression (Li et al., 2007). However, regulation may be complex and regionally specific because Smith et al. (2005) observed opposite effects of gonadectomy on KiSS1 expression in the arcuate nucleus and in the anteroventral periventricular nucleus, the former exhibiting an upregulation and the latter a downregulation. In our studies, orchidectomy resulted in a 1.5-fold increase in hippocampal KiSS1 which was, although statistically significant, rather small when compared to the 3–4 fold overall increase in the hypothalamus reported by Navarro et al. (2004). The possibility exists, however, that the effects of orchidectomy were blunted in our experiments because estrogen can be locally synthesized in the dentate gyrus (Fester et al., 2006). On the other hand, it should also be noted that estrogen directly influences diverse aspects of dentate gyrus physiology and morphology (Kim et al., 2006; Hajszan et al., 2007), and thus we cannot rule out that the effect of orchidectomy on KiSS1 mRNA was secondary to changes in excitability.

In conclusion, our studies have shown that expression of the KiSS1 gene is positively regulated by neuronal activity, and perhaps to a small extent negatively regulated by gonadal hormones. Previous studies showed that expression of GPR54 mRNA in the hippocampus is confined to dentate granule cells (Lee et al., 1996) and that kisspeptin exerts its physiological effects only in dentate granule cells and not in pyramidal neurons (Arai et al., 2005). Given that KiSS1 mRNA is expressed in the dentate gyrus (Arai et al., 2005), kisspeptin most likely acts locally in an endocrine or autocrine manner to activate its receptor GPR54, leading to greatly increased efficacy of transmission at afferent synapses and to enhanced expression of BDNF. However, the larger role of the kisspeptin system in the hippocampus remains to be determined. In the hypothalamus, GPR54 is involved in initiation of puberty, control of gonadal maturation, and regulation of the estrus cycle, and in the amygdala expression of GPR54 is highest in the anterior cortical nucleus, which receives olfactory and pheromonal input, and in the medial nucleus which exerts direct control over male sexual behavior (Hull and Dominguez, 2007). It is thus tempting to speculate that the peptide system in the hippocampus also contributes in some way to reproduction, and our finding that KiSS1 expression is influenced by gonadal hormones may support this interpretation. However, a second system-wide role of GPR54 may be to regulate cellular processes like motility, as extensively documented by the antimetastatic actions of kisspeptin (Lee et al., 1996; Lee and Welch, 1997). Given that dentate granule cells are generated from progenitor cells throughout life and have to migrate to their proper location (Cameron et al., 1993), there is thus also the intriguing possibility that kisspeptin and GPR54 are recruited to regulate neurogenesis in combination with other neurotrophic factors. Evidently, further experimentation will be needed to decide if this peptide system, in addition to its documented role in regulating local physiology, has broader functions at either the behavioral or the cell-biological level.

4. Experimental procedures

4.1. *Kainate-induced seizures*

All studies were carried out in accordance with a protocol approved by the Animal Care and Use Committee of the Southern Illinois University School of Medicine. Male Sprague–

Dawley rats (7–10 week old) from the same litter were injected intraperitoneally with kainate (8–12 mg/kg). Behavior was observed for 3 h and a seizure score was given according to a modified Racine's scale (Racine, 1972), as follows: 0, no change in behavior; 1, staring with immobility; 2, wet dog shake/head bobbing; 3, facial clonus; 4, forelimb clonus with rearing; 5, forelimb clonus with rearing and falling; 6, prolonged severe seizures with symptoms of score 5. Three hours after kainate treatment, rats were injected with phenobarbital (60 mg/kg, i.p.) to suppress seizure activity. Brain tissue was collected 1, 3 and 7 days after kainate injection, and in some cases 3 and 6 h after kainate treatment. Brains were collected into sterile ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 5, $MgCl_2$ 1, $CaCl_2$ 2, glucose 10, and HEPES 10 (pH 7.4). Coronal sections (1–1.5 mm) were cut and the hippocampus, amygdala, and periventricular hypothalamus were dissected under a stereomicroscope. Tissue samples were snap frozen with liquid nitrogen and kept at −80 °C.

4.2. *Orchidectomy*

Male Sprague–Dawley rats (49–53 days old, 250–320 g) from the same litter underwent orchidectomy performed by SIU animal care facility services. Briefly, animals were put under deep anesthesia with 4% isoflurane, and anesthesia was maintained with 2% isoflurane for the duration of the surgery. Hair on the scrotum was clipped and the skin was sterilized. An incision was made at the distal end of the scrotum and both testes were removed, after cutting of the spermatic cord. The scrotum was then sutured and animals were allowed to recover. Sham animals underwent the same procedure except for removal of the testes. Both sham and castrated animals were treated with acetaminophen (15 mg/kg) from 2 days before surgery until 5 days after surgery. Approximately 3 weeks after the surgery, animals were sacrificed and brain regions were dissected as described above.

4.3. *Organotypic slice cultures*

Organotypic hippocampal slice cultures were prepared from rats of post-natal day 10–12 according to a modified Stoppini method (Stoppini et al., 1991), as described elsewhere (Arai et al., 1996). In brief, rats were decapitated under halothane anesthesia and brains were removed into ice-cold ACSF to dissect the hippocampi. Transverse slices (400 μm) were cut with a McIlwain-type chopper. Slices from 2–4 animals were pooled and 12–16 slices were placed on the porous methylcellulose membrane of a culture plate insert (Millicell, Millipore Corp., Bedford MA) where they were maintained at the interface between atmosphere and medium. The culture medium consisted of 50% Basal Medium Eagle (Sigma, St. Louis MO, B-9638), 25% Earl's balanced salt solution (Sigma, E-7510), 25% regular horse serum (Gemini, Calabasas CA) and was supplemented with NaCl (1.17 g/L, concentrations given with regard to volume before adding serum), 0.42 g/L $NaHCO_3$, 0.029 g/L $CaCl_2$ ($2H_2O$), 0.2 g/L $MgSO_4$ (anhydrous), 0.39 g/L glutamine, 8.6 g/L glucose, 0.09 g/L ascorbic acid, 6.36 g/L HEPES (free acid), 1.3 mg/L insulin, plus 4 ml of penicillin/streptomycin (Sigma P-0781) per liter solution; the pH was adjusted at 24 °C to 7.33. After 14–28 days, slices were submitted to either electrophysiology (MED recording) or RT-PCR experiments.

4.4. *Treatment of organotypic slice cultures with kainate and picrotoxin*

Slices were exposed to 7 μM kainate for 6 h. At the end of this period, the culture insert was rinsed and transferred to a new well containing regular culture medium. Six to seven slices were harvested at various time points of kainate treatment (6 h, 1 day, 3 days and 1 week). To create a condition of chronic hyperexcitability, slices were exposed to 50 μM picrotoxin for 7 days and then submitted to analysis.

4.5. *Multi-electrode dish (MED) recording*

Slice cultures were transferred from the Millicell insert to a dish with an 8×8 array of microelectrodes that had been coated with 0.1% polyethyleneimine. Once the slices adhered to the dish (~1 h), 250 μl culture medium was added. To monitor spike activity, the dish was transferred to a recording incubator (36 °C) and activity was measured after 5 min and 10 min by recording in 40 s sweeps from all 64 electrodes (for further details see Kessler et al., 2008). Signals were amplified and digitized at 20 kHz using the MED64 system with the acquisition software Conductor (Panasonic). Activity was initially monitored for 3 days to 1 week, and slices which showed a constant activity level were then submitted to experiments with kainate and picrotoxin, using the same test conditions as for the RT-PCR experiments.

4.6. *Semi-quantitative RT-PCR*

RNA was extracted from tissue using Trizol (Invitrogen) according to the manufacturer's instructions. Briefly, a 2-fold volume of Trizol was added to a tube containing frozen brain tissue and the tissue was gently homogenized in a dounce homogenizer. The homogenates were incubated for 10 min on ice. After centrifugation, the supernatant was transferred to an Eppendorf tube. RNA was extracted with phenol–chloroform and precipitated with isopropanol/sodium acetate. The RNA was washed 2 times with 75% ethanol and desiccated in vacuo. The samples were resuspended in 10 μl of TE and their content was determined from the ratio of optical densities at 260 and 280 nm. If the sample showed a ratio lower than 1.7, the phenol–chloroform extraction was repeated. One microgram of RNA was submitted to reverse transcriptase reaction (ImProm II RT system, Promega) according to manufacturer's recommendations. A volume of 1–2 μl of the final RT product was submitted to PCR with primer pairs designed from cDNA sequences of rat KiSS1, GPR54, BDNF and actin. The primer sequences were: for *KiSS1*: 5′-TGG CAC CTG TGG TGA ACC CTG AAC-3′, 3′-CAC ACG GTG GGC GTC AGC GGA CTA-5′ (Navarro et al., 2004); for *GPR54*: 5′-CAG CTG TTC CTG GTG CTT CAA-3′, 3′-G TAC AGG ATG TCG TTA AGA CGC-5′; for *BDNF*: 5′-AGG ACG CGG ACT TGT ACA CT-3′, 3′-CAA TGT GCT TCC TTC CGA CG-5′; for *actin*: 5′-AAG ATC CTG ACC GAG CGT GG-3′, 3′-G GAG ATA CGG TTG TGT CAC GAC-5′. The amplified sequence for KiSS1 spans an intron. No genomic DNA contamination was observed.

The original RT product (1 μl for actin, BDNF, GPR54 and 4 μl for KiSS1) and a 5-time diluted sample were submitted to PCR. If the diluted sample did not show one fifth of the signal of the original mixture, another PCR was performed with a reduced cycle number. Typical PCR conditions were: an initial incubation at 95 °C for 5 min, then cycling at 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1.5 min, and a final stage of 72 °C for 2 min. Optimal cycle numbers for each mRNA of interest were: actin 18 cycles, BDNF 24 cycles, GPR54 32 cycles and KiSS1 37 cycles. The PCR products were submitted to PAGE gel electrophoresis and stained with ethidiumbromide. The optical signal was captured with a CCD camera and analyzed using Image J. KiSS1, GPR54 and BDNF were normalized to the corresponding signal for actin. The size of the PCR product was 202 bp for KiSS1, 111 bp for GPR54, 335 bp for BDNF, and 327 bp for actin.

4.7. *Data analysis*

In each experiment, slice cultures and tissues from animals treated with kainate or picrotoxin were processed in parallel with one or several control samples and normalized to the latter. Statistical significance of the averaged data was then assessed using a 1-sample *t*-test, using Graphpad Prism. Data are generally presented as mean and s.e.m.

4.8. *Drugs and peptide*

For i.p. injection, kainic acid (Sigma) was dissolved at 5–6 mg/ml in 0.9% NaCl and the pH was adjusted to ~7.5. For treatment of organotypic slice cultures, stock solutions were prepared of 1 mM kainate in 0.9% NaCl and 25 mM picrotoxin in DMSO. The stock solutions were diluted to the final concentration with culture medium. Rat kisspeptin-10 (YNWNSFGLRYamide) was obtained from Phoenix Pharmaceuticals (Burlingame, CA).

Acknowledgments

This work was supported by a grant from the Whitehall Foundation (2007-05-119) and by funds from the Central Research Committee of the Southern Illinois University School of Medicine. We would like to thank Nancy Johnston, DVM, and Andrea M. Frazier from the animal care facility for providing surgical services for orchidectomy.

REFERENCES

Arai, A.C., Kessler, M., Rogers, G., Lynch, G., 1996. Effects of a memory enhancing drug on AMPA receptor currents and synaptic transmission in hippocampus. J. Pharmacol. Exp. Ther. 278, 627–638.

Arai, A.C., Xia, Y.F., Suzuki, E., Kessler, M., Civelli, O., Nothacker, H.P., 2005. Cancer metastasis-suppressing peptide metastin upregulates excitatory synaptic transmission in hippocampal dentate granule cells. J. Neurophysiol. 94, 3648–3652.

Bilban, M., Ghaffari-Tabrizi, N., Hintermann, E., Bauer, S., Molzer, S., Zoratti, C., Malli, R., Sharabi, A., Hiden, U., Graier, W., Knöfler, M., Andreae, F., Wagner, O., Quaranta, V., Desoye, G., 2004. Kisspeptin-10, a KiSS-1/metastin-derived decapeptide, is a physiological invasion inhibitor of primary human trophoblasts. J. Cell Sci. 117, 1319–1328.

Brailoiu, G.C., Dun, S.L., Ohsawa, M., Yin, D., Yang, J., Chang, J.K., Brailoiu, E., Dun, N.J., 2005. KiSS-1 expression and metastin-like immunoreactivity in the rat brain. J. Comp. Neurol. 481, 314–329.

Cameron, H.A., Woolley, C.S., McEwen, B.S., Gould, E., 1993. Differentiation of newly born neurons and glia in the dentate gyrus of the adult rat. Neuroscience 56, 337–344.

Castellano, J.M., Gaytan, M., Roa, J., Vigo, E., Navarro, V.M., Bellido, C., Dieguez, C., Aguilar, E., Sánchez-Criado, J.E., Pellicer, A., Pinilla, L., Gaytan, F., Tena-Sempere, M., 2006. Expression of KiSS-1 in rat ovary: putative local regulator of ovulation? Endocrinology 147, 4852–4862.

Conner, J.M., Lauterborn, J.C., Yan, Q., Gall, C.M., Varon, S., 1997. Distribution of brain-derived neurotrophic factor (BDNF) protein and mRNA in the normal adult rat CNS: evidence for anterograde axonal transport. J. Neurosci. 17, 2295–2313.

Coulter, D.A., Carlson, G.C., 2007. Functional regulation of the dentate gyrus by GABA-mediated inhibition. Prog. Brain Res. 163, 235–243.

del Cerro, S., Arai, A., Kessler, M., Bahr, B.A., Vanderklish, P., Rivera, S., Lynch, G., 1994. Stimulation of NMDA receptors activates calpain in cultured hippocampal slices. Neurosci. Lett. 167, 149–152.

de Roux, N., Genin, E., Carel, J.C., Matsuda, F., Chaussain, J.L., Milgrom, E., 2003. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. Proc. Natl. Acad. Sci. U. S. A. 100, 10972–10976.

Fester, L., Ribeiro-Gouveia, V., Prange-Kiel, J., von Schassen, C., Böttner, M., Jarry, H., Rune, G.M., 2006. Proliferation and apoptosis of hippocampal granule cells require local oestrogen synthesis. J. Neurochem. 97, 1136–1144.

Funes, S., Hedrick, J.A., Vassileva, G., Markowitz, L., Abbondanzo, S., Golovko, A., Yang, S., Monsma, F.J., Gustafson, E.L., 2003. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. Biochem. Biophys. Res. Commun. 312, 1357–1363.

Gall, C., 1988. Seizures induce dramatic and distinctly different changes in enkephalin, dynorphin, and CCK immunoreactivities in mouse hippocampal mossy fibers. J. Neurosci. 8, 1852–1862.

Gall, C.M., 1993. Seizure-induced changes in neurotrophin expression: implications for epilepsy. Exp. Neurol. 124, 150–166.

Gottsch, M.L., Cunningham, M.J., Smith, J.T., Popa, S.M., Acohido, B.V., Crowley, W.F., Seminara, S., Clifton, D.K., Steiner, R.A., 2004. A role for kisspeptins in the regulation of gonadotropin secretion in the mouse. Endocrinology 145, 4073–4077.

Hajszan, T., Milner, T.A., Leranth, C., 2007. Sex steroids and the dentate gyrus. Prog. Brain Res. 163, 399–415.

Han, S.K., Gottsch, M.L., Lee, K.J., Popa, S.M., Smith, J.T., Jakawich, S.K., Clifton, D.K., Steiner, R.A., Herbison, A.E., 2005. Activation of gonadotropin-releasing hormone neurons by kisspeptin as a neuroendocrine switch for the onset of puberty. J. Neurosci. 25, 11349–11356.

Hull, E.M., Dominguez, J.M., 2007. Sexual behavior in male rodents. Horm. Behav. 52, 45–55.

Isackson, P.J., Huntsman, M.M., Murray, K.D., Gall, C.M., 1991. BDNF mRNA expression is increased in adult rat forebrain after limbic seizures: temporal patterns of induction distinct from NGF. Neuron 6, 937–948.

Kessler, M., Kiliman, B., Humes, C., Arai, A.C., 2008. Spontaneous activity in Purkinje cells: multi-electrode recording from organotypic cerebellar slice cultures. Brain Res. 1218, 54–69.

Kim, M.T., Soussou, W., Gholmieh, G., Ahuja, A., Tanguay, A., Berger, T.W., Brinton, R.D., 2006. 17beta-Estradiol potentiates field excitatory postsynaptic potentials within each subfield of

the hippocampus with greatest potentiation of the associational/commissural afferents of CA3. Neuroscience 141, 391–406.
Kotani, M., Detheux, M., Vandenbogaerde, A., Communi, D., Vanderwinden, J.M., Le Poul, E., Brezillon, S., Tyldesley, R., Suarez-Huerta, N., Vandeput, F., Blanpain, C., Schiffmann, S.N., Vassart, G., Parmentier, M., 2001. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. J. Biol. Chem. 276, 34631–34636.
Koyama, R., Yamada, M.K., Fujisawa, S., Katoh-Semba, R., Matsuki, N., Ikegaya, Y., 2004. Brain-derived neurotrophic factor induces hyperexcitable reentrant circuits in the dentate gyrus. J. Neurosci. 24, 7215–7224.
Lee, J.H., Welch, D.R., 1997. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. Cancer Res. 57, 2384–2387.
Lee, J.H., Miele, M.E., Hicks, D.J., Phillips, K.K., Trent, J.M., Weissman, B.E., Welch, D.R., 1996. KiSS-1, a novel human malignant melanoma metastasis-suppressor gene. J. Natl. Cancer Inst. 88, 1731–1737.
Lee, D.K., Nguyen, T., O'Neill, G.P., Cheng, R., Liu, Y., Howard, A.D., Coulombe, N., Tan, C.P., Tang-Nguyen, A.T., George, S.R., O'Dowd, B.F., 1999. Discovery of a receptor related to the galanin receptors. FEBS Lett. 446, 103–107.
Li, D., Mitchell, D., Luo, J., Yi, Z., Cho, S.G., Guo, J., Li, X., Ning, G., Wu, X., Liu, M., 2007. Estrogen regulates KiSS1 gene expression through estrogen receptor alpha and SP protein complexes. Endocrinology 148, 4821–4828.
Masui, T., Doi, R., Mori, T., Toyoda, E., Koizumi, M., Kami, K., Ito, D., Peiper, S.C., Broach, J.R., Oishi, S., Niida, A., Fujii, N., Imamura, M., 2004. Metastin and its variant forms suppress migration of pancreatic cancer cells. Biochem. Biophys. Res. Comm. 315, 85–92.
Messaoudi, E., Bardsen, K., Srebro, B., Bramham, C.R., 1998. Acute intrahippocampal infusion of BDNF induces lasting potentiation of synaptic transmission in the rat dentate gyrus. J. Neurophysiol. 79, 496–499.
Monaghan, D.T., Yao, D., Cotman, C.W., 1984. Distribution of [3H] AMPA binding sites in rat brain as determined by quantitative autoradiography. Brain Res. 324, 160–164.
Muir, A.I., Chamberlain, L., Elshourbagy, N.A., Michalovich, D., Moore, D.J., Calamari, A., Szekeres, P.G., Sarau, H.M., Chambers, J.K., Murdock, P., Steplewski, K., Shabon, U., Miller, J.E., Middleton, S.E., Darker, J.G., Larminie, C.G., Wilson, S., Bergsma, D.J., Emson, P., Faull, R., Philpott, K.L., Harrison, D.C., 2001. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. J. Biol. Chem. 276, 28969–28975.
Nadler, J.V., 1981. Kainic acid as a tool for the study of temporal lobe epilepsy. Life Sci. 29, 2031–2042.
Navarro, V.M., Castellano, J.M., Fernandez-Fernandez, R., Barreiro, M.L., Roa, J., Sanchez-Criado, J.E., Aguilar, E., Dieguez, C., Pinilla, L., Tena-Sempere, M., 2004. Developmental and hormonally regulated messenger ribonucleic acid expression of KiSS-1 and its putative receptor, GPR54, in rat hypothalamus and potent luteinizing hormone-releasing activity of KiSS-1 peptide. Endocrinology 145, 4565–4574.
Ohtaki, T., Shintani, Y., Honda, S., Matsumoto, H., Hori, A., Kanehashi, K., Terao, Y., Kumano, S., Takatsu, Y., Masuda, Y., Ishibashi, Y., Watanabe, T., Asada, M., Yamada, T., Suenaga, M., Kitada, C., Usuki, S., Kurokawa, T., Onda, H., Nishimura, O., Fujino, M., 2001. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. Nature 411, 613–617.
Pielecka-Fortuna, J., Chu, Z., Moenter, S.M., 2008. Kisspeptin acts directly and indirectly to increase GnRH neuron activity and its effects are modulated by estradiol. Endocrinology 149, 1979–1986.
Poulsen, F.R., Lauterborn, J., Zimmer, J., Gall, C.M., 2004. Differential expression of brain-derived neurotrophic factor transcripts after pilocarpine-induced seizure-like activity is related to mode of Ca^{2+} entry. Neuroscience 126, 665–676.
Racine, R.J., 1972. Modification of seizure activity by electrical stimulation II. Motor seizure. Electroencephalogr. Clin. Neurophysiol. 32, 281–294.
Routbort, M.J., Bausch, S.B., McNamara, J.O., 1999. Seizure, cell death, and mossy fiber sprouting in kainic acid-treated organotypic hippocampal cultures. Neuroscience 94, 755–765.
Scharfman, H.E., 2002. Epilepsy as an example of neural plasticity. Neuroscientist 8, 154–173.
Seminara, S.B., Messager, S., Chatzidaki, E.E., Thresher, R.R., Acierno Jr, J.S., Shagoury, J.K., Bo-Abbas, Y., Kuohung, W., Schwinof, K.M., Hendrick, A.G., Zahn, D., Dixon, J., Kaiser, U.B., Slaugenhaupt, S.A., Gusella, J.F., O'Rahilly, S., Carlton, M.B., Crowley Jr, W.F., Aparicio, S.A., Colledge, W.H., 2003. The GPR54 gene as a regulator of puberty. N. Engl. J. Med. 349, 1614–1627.
Semple, R.K., Achermann, J.C., Ellery, J., Farooqi, I.S., Karet, F.E., Stanhope, R.G., O'Rahilly, S., Aparicio, S.A., 2005. Two novel missense mutations in GPR54 in a patient with hypogonadotropic hypogonadism. J. Clin. Endocrin. Metab. 90, 1849–1855.
Shimono, K., Brucher, F., Granger, R., Lynch, G., Taketani, M., 2000. Origins and distribution of cholinergically induced beta rhythms in hippocampal slices. J. Neurosci. 20, 8462–8473.
Smith, J.T., Dungan, H.M., Stoll, E.A., Gottsch, M.L., Braun, R.E., Eacker, S.M., Clifton, D.K., Steiner, R.A., 2005. Differential regulation of KiSS-1 mRNA expression by sex steroids in the brain of the male mouse. Endocrinology 146, 2976–2984.
Sperk, G., 1994. Kainic acid seizures in the rat. Prog. Neurobiol. 42, 1–32.
Stoppini, L., Buchs, P.A., Muller, D., 1991. A simple method for organotypic cultures of nervous tissue. J. Neurosci. Methods 37, 173–182.