Fig. 2. Histograms of the data recorded in Fig. 1. Maximal amplitude of the curves shows significant ($p < 0.01$) concentration-dependent elevation (a). The areas-under-curve representing the net calcium influx also demonstrates significant ($p < 0.01$) concentration-dependent increase in the calcium content (b). Both histograms were calculated after baseline subtraction. The areas-under-curve data were then normalized by setting the calculated data recorded at application of 250 nM peptide as 100%. The mean ± S.E.M. values of the "means of maxima" were calculated by first determining the maximum amplitude of calcium recording of each cell within the same treatment. Then the calculation of the mean and the S.E.M. was carried out.

measurements demonstrated that 250 nM PL37-MAP evoked inward ion current pulses in GT1-7 cells (Fig. 5a). Amplitude of the pulses was 304 ± 69 pA ($n = 12$). Magnocellular neurons of the supraoptic (SON) and paraventricular (PVN) nuclei of rat brain slices also responded with inward current pulses (SON: 1043 ± 129 pA, $n = 9$; PVN: 1467 ± 219 pA, $n = 10$) to the PL37-MAP (2 μM) administration (Fig. 5b and c). Response of GT1-7 cells to the peptide treatment suggested that GnRH neurons in the brain slice might also react to the PL37-MAP.

Therefore, GnRH-GFP neurons of the mice were also treated with PL37-MAP (2 μM) and, indeed, the peptide could trigger inward current pulses in these cells (580 ± 82 pA; $n = 8$; Fig. 5d). In contrast, non-neuroendocrine neurons of the anterior hypothalamic area (AHA; $n = 8$) did not respond to the PL37-MAP administration (Fig. 5e) suggesting that these cells do not possess C5aR in their membrane. After 5 min PL37-MAP treatment the peptide was washed out and 28% of the cells recovered, whereas in the other neurons the inward current
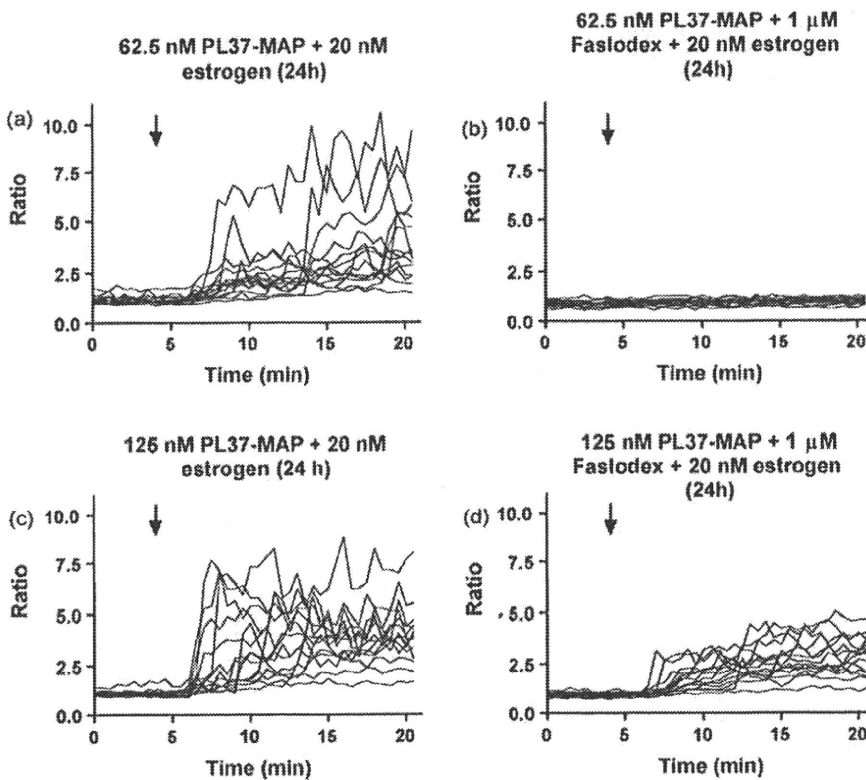

Fig. 3. Calcium imaging measurements of GT1-7 cells pretreated with E2 and then treated with two concentrations of the PL37-MAP. When the cells were pretreated with E2, even low concentration of the peptide (62.5 nM) evoked calcium influx (a). Higher concentration of the peptide (125 nM) also elicited calcium influx (c). However, when E2 was co-applied with the estrogen receptor blocker Faslodex, response of the cells was eliminated at low concentration of PL37-MAP (b) and was attenuated at 125 nM peptide (d). Arrow shows application of the peptide.
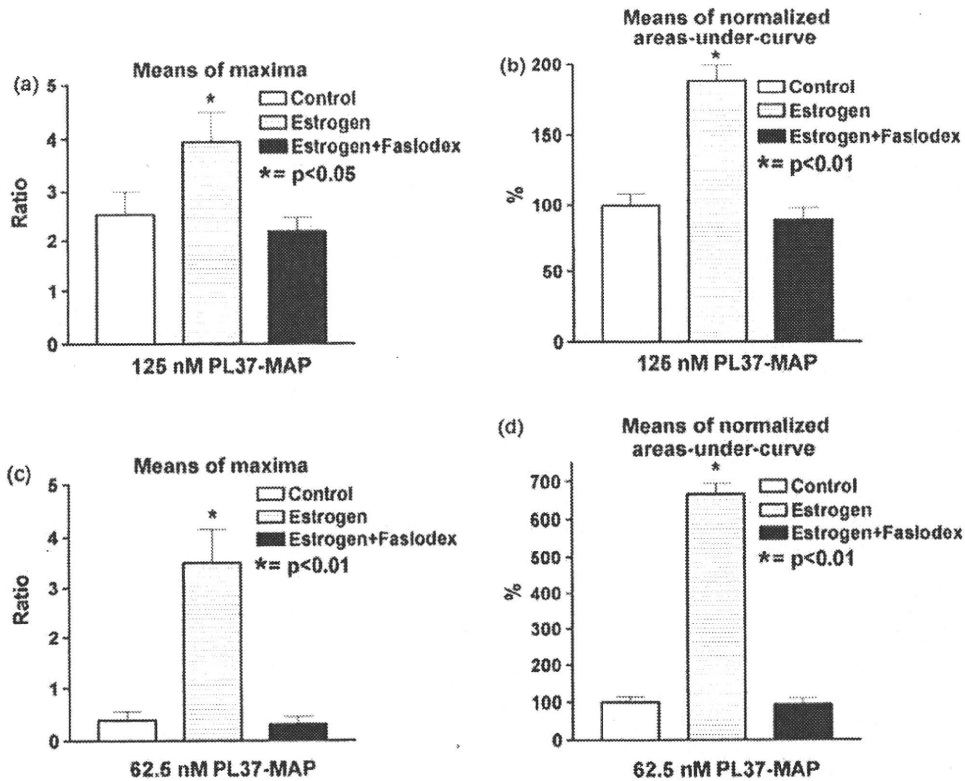
Fig. 4. Histograms of the data recorded in Fig. 3. E2 treatment significantly elevated the maximal amplitude of the recorded curves both at 125 nM (a, $p < 0.05$) and 62.5 nM PL37-MAP (c, $p < 0.01$). Faslodex blocked the elevation of the amplitude evoked by E2 (a and c). The areas-under-curve data show similar tendency, E2 pretreatment elevated the PL37-MAP elicited response of the cells significantly ($p < 0.01$) at both concentrations of PL37-MAP (b and d). All of the histograms were calculated using baseline correction and the areas-under-curve data were normalized by setting the control measurements as 100%. The mean ± S.E.M. values of the "means of maxima" were calculated by determining the maximum amplitude of calcium recording of each cell upon a certain treatment and then calculation of the mean and the S.E.M. was carried out.

reached a plateau (GT1-7 cells: 225 ± 54 pA; SON: 760 ± 172 pA; PVN: 1117 ± 214 pA). The extracellularly applied calcium channel blocker $CoCl_2$ (0.5 mM) abolished the current evoked by the PL37-MAP suggesting that the triggered inward pulses were due to calcium influx in the PVN (Fig. 5f). $CoCl_2$ also eliminated the evoked current in the GT1-7, SON and GnRH-GFP neurons (not shown). Pretreatment of the brain slice with C5a (5 μg/ml) in the extracellular solution significantly decreased the amplitude of the PL37-MAP-triggered inward current in the PVN (624 ± 79 pA, $n = 8$; $p < 0.05$; Fig. 5g) suggesting that the current was due to the activation of the C5aR. The PL37-MAP evoked current was diminished by the C5a-pretreatment in the GT1-7 cells (167 ± 45 pA; $n = 13$), the neurons of the SON (604 ± 110 pA; $n = 8$) and the GnRH-GFP cells (352 ± 57 pA; $n = 8$) as well ($p < 0.05$; not shown).

Before treatments with the PL37-MAP peptide, the cells were identified as neurons by applying +10 pA current with −10 pA prepulse in current clamp mode. The current injected has evoked action potential in the magnocellular cells of the SON and PVN (Fig. 6a and b). The protocol for the injected current is graphed in the inset of Fig. 6a. The action potentials presented no low threshold spike (LTS). In addition, a voltage gated transient outward rectifying (A-type) potassium ion current could be recorded in the neurons of the PVN (Fig. 6c). The voltage command protocol is in the inset: prepulse

parameters were 20 ms and −90 mV whereas the pulses (30 ms) stepped from −80 mV up to +40 mV with steps of 10 mV. Lack of LTS in the SON and PVN and presence of the transient outward potassium current in the PVN suggested that these cells were neurosecretory magnocellular neurons (Hoffman et al., 1991; Tasker and Dudek, 1991). The GnRH-GFP cells also presented action potentials when current command was applied (Fig. 6d) demonstrating that the cells responding to the C5aR-agonist treatment were neurons. The injected current evoked action potentials in the cells of the AHA showing that these cells were neurons, too (Fig. 6e).

In order to demonstrate the action of estrogen in the neurons of the brain slice, GnRH-GFP neurons were treated with 2 μM PL37-MAP peptide in hypothalamic slices obtained from ovariectomized (OVX) and E2-substituted (OVX + E2) animals. PL37-MAP evoked inward pulses in the GnRH neurons of the OVX + E2 mice (610 ± 76 pA; $n = 8$) (Fig. 7a). The GnRH neurons of the OVX mice responded to the peptide with small inward current pulses (102 ± 45 pA; $n = 8$) (Fig. 7b) with significantly lower amplitude than in the neurons of the OVX + E2 mice ($p < 0.01$).

3.3. Real-time PCR experiments

To potentially explain the observed effect of E2 on the current evoked by the C5aR-agonist peptide, we examined
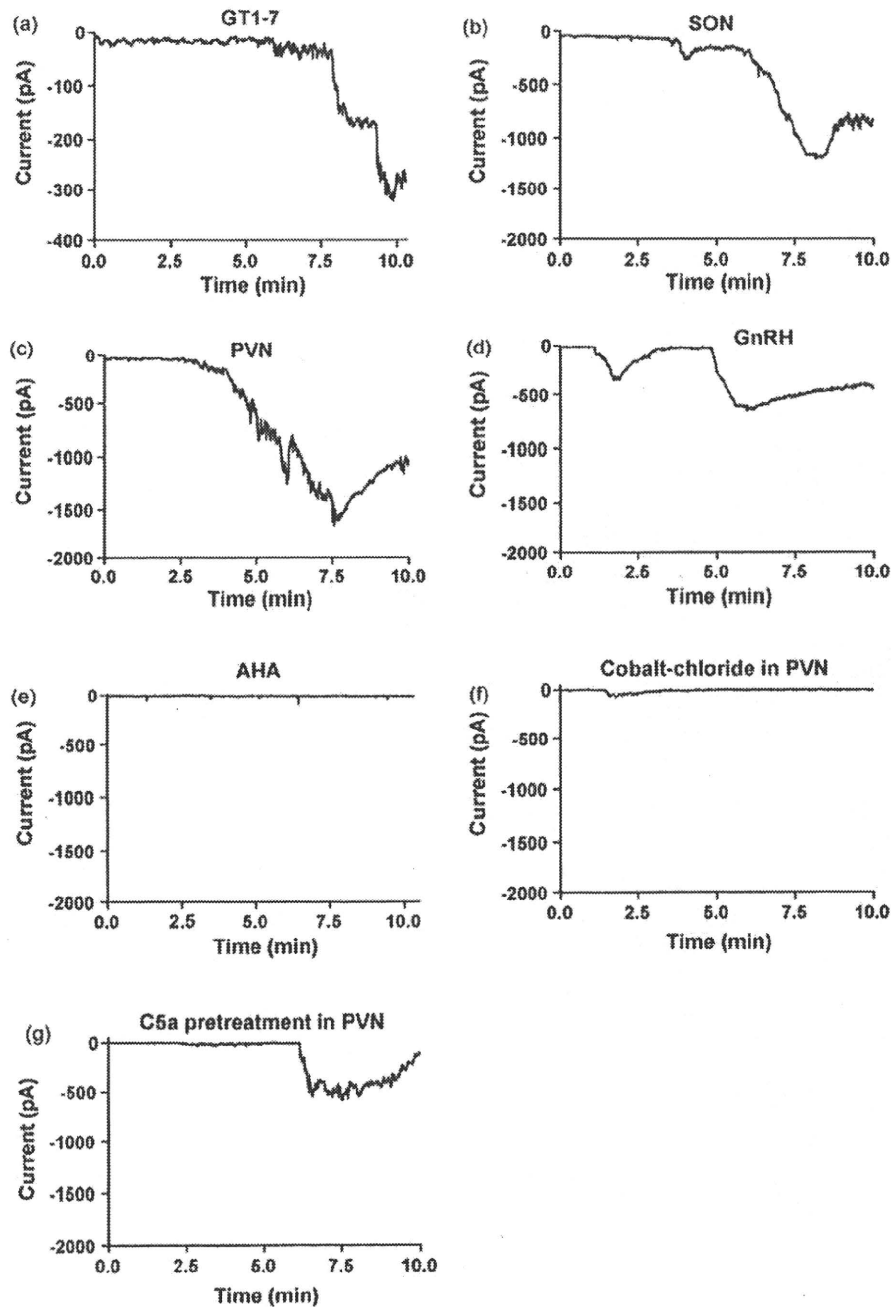
Fig. 5. The C5a-agonist peptide evoked inward current pulses, as determined by whole cell clamp electrophysiology in various hypothalamic cells. GT1-7 cells (a), magnocellular cells of the supraoptic (SON, b) and paraventricular nuclei (PVN, c) and GnRH cells (d) responded to PL37-MAP peptide with inward ion current. Cells of the anterior hypothalamic area (AHA) did not respond to the peptide administration (e). Calcium channel blocker $CoCl_2$ eliminated the evoked current in the PVN cells (f). Pretreatment of the brain slice with C5a diminished the amplitude of the inward current triggered by PL37-MAP in the PVN cells (g).

the expression of the C5aR in GT1-7 cells with real-time PCR. The expression ratio of C5aR and hypoxanthine-guanine phosphoribosil transferase (HPRT) genes in each sample (normalized data) was determined and compared (Fig. 8). In non-treated, control cells only low level of expression of the C5aR was detectable. Treatment of the cells with 20 nM E2 for 30 min, 2 h and 8 h resulted in mRNA signal intensities that did not significantly differ from those of the control. Nevertheless, treatment for 24 h caused

a marked elevation in the C5aR mRNA level—reaching a significant 57-fold level (56.9 ± 32.44) compared to the signal intensities detected in non-treated cultures ($p < 0.01$). This showed that E2 induced C5aR expression and this process required E2 treatment for longer than 8 h. An additional day of E2 treatment (48 h) resulted in a decrease of the gene product. This indicated that the E2-dependent induction was transient in spite of the continuous presence of E2.
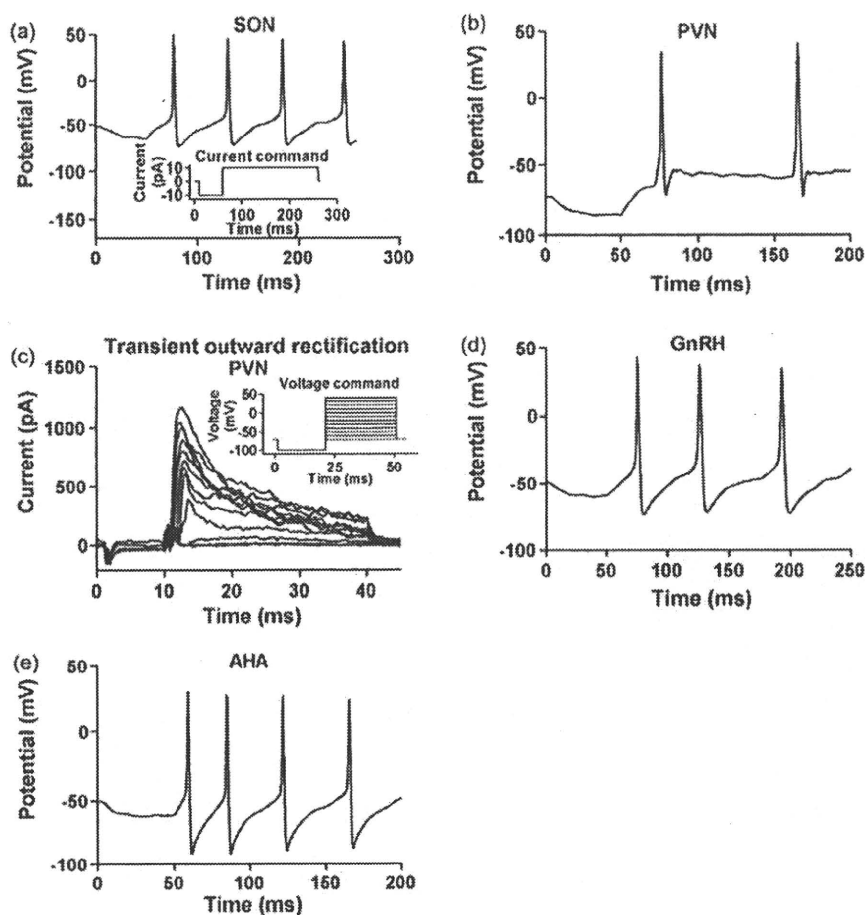
Fig. 6. Identifying the neuronal phenotype of the recorded cells shown in Fig. 5. The recorded cells of the SON, PVN and AHA in Fig. 5 were identified as neurons by their characteristic location and morphology and by the evoked action potentials using current clamp method (SON, a; PVN, b; AHA, e). Current clamp protocol details are given in the inset of (a). In addition, cells of the PVN were identified as magnocellular neurosecretory neurons by the presence of the transient outward rectification current (c) using voltage command seen in the inset. GnRH neurons were identified by their GFP fluorescence and the evoked action potentials (d).

4. Discussion

Few reports have suggested interactions between the complement system and the hypothalamus, particularly in the regulation of fever (Sehic et al., 1998; Blatteis and Sehic, 1998). A direct evidence for the central actions of C5aR was obtained following examination of the binding specificity of

C5a injected into the hypothalamus, which suggested a presynaptic action for this anaphylatoxin (Schupf et al., 1989). The view that hypothalamic neurons express C5aR is also supported by the finding that direct injection of C5a into the perifornical region of the lateral hypothalamus of the rat changed eating and drinking behavior. C5a injection into this area stimulated eating in the satiated rats
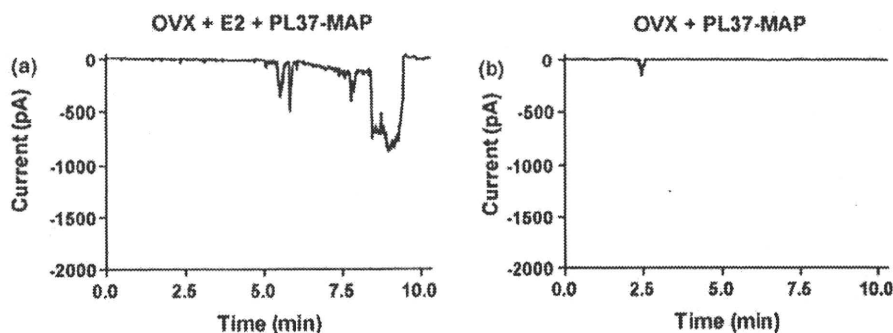

Fig. 7. The PL37-MAP peptide evoked inward current pulses in the GnRH-GFP neurons of the brain slice obtained from ovariectomized and E2 substituted (OVX + E2, a) and in ovariectomized (OVX, b) female mice. Amplitude of the triggered pulses in OVX brain slices was lower than in OVX + E2 slices showing that E2 increased the response elicited by PL37-MAP.
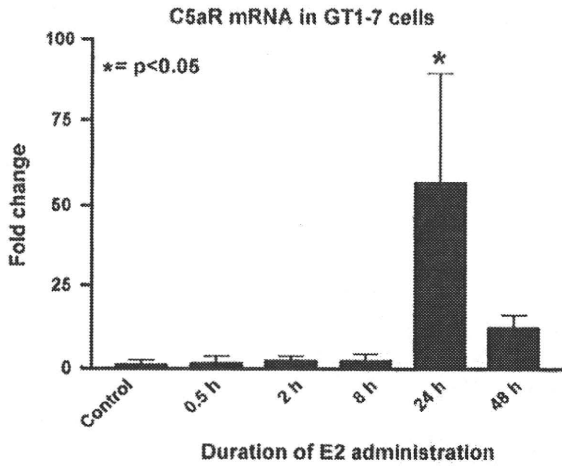
Fig. 8. Real-time PCR measurement of the E2-dependency of the expression of C5aR in GT1-7 cells. Treatment of the cells with E2 for 24 h elevated expression of C5aR followed by a decrease after 48 h.

and decreased carbamyl-choline-induced drinking (Williams et al., 1985).

Hippocampal and cortical pyramidal cells, cerebellar Purkinje cells and certain ventral and dorsal horn neurons of the spinal cord were earlier shown to bear C5aR (Farkas et al., 2003; Stahel et al., 1997a,b; VanBeek et al., 2000; O'Barr et al., 2001). Our experiments have now revealed that several neurosecretory cell types in the rat and mouse hypothalami – a brain area which has not been thoroughly investigated for expression of neuronal C5aR so far – also expressed complement C5aR. Magnocellular neurons of the supraoptic and paraventricular nuclei and GnRH-producing neurons responded to administration of the C5aR-agonist peptide demonstrating the presence of the functional C5aR in their membrane. However, neurons tested from the anterior hypothalamic area, which are not neurosecretory cells, were not activated by PL37-MAP suggesting that not all hypothalamic neurons bear C5aR. This result is supported by other works reporting that C5aR was only present in specific groups of neurons in the cortex and the hippocampus (Farkas et al., 2003; O'Barr et al., 2001).

Expression of the C5aR in the neurosecretory cells of the SON and PVN indicates that the inflammatory signals triggered inside the CNS might modulate the hormonal responses by these neurons. Although inflammatory signals generated in the periphery could also alter the hormonal response, the access of such signals to neurons is confined to brain regions lacking the blood–brain barrier. These include the organum vasculosum laminae terminalis (OVLT) and the median eminence. In addition, modulation of neuronal functions by the peripheral C5a is also limited by the short half-life of the circulating C5a. Nevertheless, modulation of the hormonal responses by the inflammatory signals is supported by the lipopolisaccharide (LPS)-evoked increase in c-fos immunoreactivity in neurons of the SON and PVN, followed by elevated plasma levels of vasopressin and oxytocin (de Carvalho Borges et al., 2006). The effect of LPS might be mediated via the production of cytokines

such as tumor necrosis factor α (TNF-α), interleukins IL-1 and IL-6 (Haddad et al., 2002) which induce nuclear c-fos expression in the magnocellular neurons of the PVN and SON (Xia and Krukoff, 2003). However, TNF-α, the first of the cytokines to appear is not detectable until 30 min or even longer after the LPS challenge (Perlik et al., 2005), whereas vasopressin has already reached its highest level by this time (Giusti-Paiva et al., 2002). Nevertheless, a faster signal can be triggered via activation of the complement system. LPS in blood or tissue activates the alternative pathway of the C almost immediately. This results in the appearance of the complement cascade elements, including C5a, in 2–3 min (Giusti-Paiva et al., 2002; Blatteis, 2006). Activation of C5aR can elevate calcium content and c-fos expression via a G-protein-dependent pathway in neurons (Farkas et al., 1998a,b). In addition, C5a activation can elicit cytokine release from various cell types (Riedemann et al., 2004). In case of inflammation, C5a can be generated either locally in the brain (Terai et al., 1997; Gasque et al., 2000; Strohmeyer et al., 2000) or could enter the hypothalamus via structures lacking the blood–brain barrier (OVLT and median eminence, in case of the hypothalamus). Therefore, C5a bound to the C5aR may be considered as a putative early phase mediator in the hormonal response evoked by an inflammatory signal in the magnocellular neurons of the SON and PVN leading to increased vasopressin and oxytocin contents in the plasma. However, the validation of this hypothesis requires further experiments.

The C5aR was found in GnRH-producing neurons, too, indicating that C5a/C5aR might contribute to the inflammation-related pathology of the GnRH system. These neurons are located in the preoptic area around the OVLT suggesting that not only local stimuli but also peripherally induced C5a might reach these cells. Indeed, the reproductive axis is heavily suppressed under inflammation involving inhibition of GnRH release (He et al., 2003). Inflammatory stress can even disrupt the ovarian cyclicity (Karsch et al., 2002). These results support the idea that C5aR may interfere with reproduction via altering the function of GnRH neurons.

Our present data show that the response of the GnRH neurons to the activation of C5aR can be modified by estrogen. The calcium influx evoked by administration of the C5aR-agonist peptide was amplified upon E2 treatment. In addition, the effect of this hormone might be mediated via the estrogen receptor because the estrogen receptor antagonist Faslodex inhibited the amplification. Electrophysiological recordings revealed that E2 elevated the amplitude of the inward ion current recorded upon applying PL37-MAP to GnRH neurons. These results suggest that elements of the C and estrogen could interact in the GnRH neurons. Similar interactions between estrogen and certain elements of the C have already been reported in the uterus where complement C3 is considered as one of the most sensitive marker of estrogenic effects (Christoffel et al., 2006). The significance of amplification of the C5aR-mediated response by estrogen in GnRH neurons has not been elucidated yet. Recent results, however, indicate that estrogen is required for a proper immune response to bacterial and viral pathogens in the brain of female mice (Soucy et al., 2005).

855

In addition to its function in mediating the immune response, C5aR is also considered to play a role in neurodegeneration (Farkas et al., 2003; Woodruff et al., 2006). It is known that persistent high calcium concentration could trigger detrimental effects in neurons. Our earlier results demonstrated that non-physiological activation of the C5aR related with high calcium influx could evoke apoptotic signals (Farkas et al., 1998a,b). Although according to our present knowledge there is no reason to suppose that activation of the C5aR by C5a under physiological condition could elicit harmful outcome, the earlier data mentioned above suggest that the persistent high calcium influx evoked by PL37-MAP in the hypothalamic neurons could finally result in a degenerative process. This also raises the possibility that chronic release of C5a or presence of its fragments under pathological conditions – which could be modeled by presence of the PL37-MAP – could elicit detrimental effects in the neurosecretory neurons of the hypothalamus. In addition, the C5aR is associated with multiple signal transduction pathways including the ones that trigger apoptosis in neurons and thymocytes (Riedemann et al., 2002; Farkas et al., 1998a,b). Furthermore, apoptotic signal is related to an elevated expression of the C5aR (Riedemann et al., 2002). Therefore, overamplification of the C5aR signal could be detrimental. The real-time PCR measurements presented here have shown that estrogen increased the expression of the C5aR in the GnRH-producing GT1-7 neurons. These data are in harmony and strengthen those of our patch clamp and calcium imaging studies showing that estrogen is capable of amplifying the C5aR-mediated signal. Therefore, from our results we predict that under chronic inflammatory conditions estrogen could be involved in a positive feedback loop amplifying the signals – including calcium influx – evoked by activation of the C5aR. Deleterious effects of estrogen have already been shown in GT1-7 cells where it enhanced glutamate-induced neuro-toxicity (Yang et al., 2003). Other authors also claimed that the impact of estrogen highly depends on the circumstances (type and concentration of estrogen, exposure time, gender, age, etc.) under which it acts (Nordell et al., 2003; Sohrabji, 2005; Chen et al., 2006). Further experiments are required to investigate such a role of estrogen in the future.

In conclusion, we have shown that magnocellular neurons of the supraoptic and paraventricular nuclei and GnRH-producing neurons of the hypothalamus express functional receptor for the C5a. In addition, we have revealed that estrogen treatment modulates the C5aR-mediated signal and the action of estrogen could be related to the estrogen receptor. Furthermore, we have provided evidence for the up-regulation of the C5aR transcript by estrogen. The significance of these findings relates to the better understanding of the inflammatory and neurodegenera-tory diseases of the hypothalamus and the related neuroendo-crine and autonomic compensatory responses.

Acknowledgements

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