Venlafaxine exhibits an anti-inflammatory effect in an inflammatory co-culture model

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Abstract
Growing evidence indicates immunoregulatory effects of various antidepressants. Through the interaction of the nervous and immune systems, the norepinephrine–serotonin system was shown to modulate inflammatory CNS diseases. Thus, we examined the norepinephrine–serotonin reuptake inhibitor venlafaxine in an astroglia–microglia co-culture model which allows mimicking of an inflammatory milieu by increasing the cultured microglial fraction. Astrocytic membrane resting potential and intercellular coupling, two markers becoming severely impaired under inflammation, were assessed with the patch-clamp technique. We measured IL-6, IL-10, IFN-γ and TGF-β concentrations and analysed phenotypic changes of microglia. We found (i) a reversal of the inflammation-induced depolarization effect on the membrane resting potential, (ii) an augmentation of TGF-β release with a concomitant reduction in the secretion of pro-inflammatory IL-6 and IFN-γ, and (iii) a significant change of microglial phenotype from activated to resting morphology. Our data clearly indicate anti-inflammatory properties of venlafaxine which might be a result of monoamine-mediated immunomodulation.

Introduction
In the past years, several studies uncovered immunoregulatory effects of antidepressant agents (e.g. Maes, 2001). Venlafaxine, fluoxetine and imipramine were found to have negative immunoregulatory effects by suppressing the interferon-γ–interleukin-10 (IFN-γ–IL-10) production ratio in whole-blood cells (Kubera et al., 2001). Further studies reported decreasing pro-inflammatory and increasing anti-inflammatory cytokine levels (Kenis and Maes, 2002; Xia et al., 1996) under antidepressant treatments. Recently, amitryptiline was shown to inhibit interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) production in rat mixed glial and microglial cultures (Obuchowicz et al., 2006).

Changes of the serotonin (5-HT) and norepinephrine (NE) transmitter systems have been reported in the pathogenesis of affective disorders which are efficiently treated with selective 5-HT and/or NE reuptake inhibitors. Both transmitter systems have been suggested to serve as mediators of bi-directional interactions between the nervous and the immune systems (Felten et al., 1992; Mossner and Lesch, 1998). For instance, 5-HT receptor-deficient transgenic mice when challenged with experimental allergic encephalomyelitis (EAE) revealed a reduction of inflammatory infiltrates in the CNS and of the neuroantigen-specific production of IFN-γ in splenocytes (Hofstetter et al., 2005). Further studies showed a suppression of clinical signs in experimental allergic neuritis when animals were treated with selective serotonin reuptake inhibitors (Bengtsson et al., 1992).

Earlier investigations on the effect of NE showed a suppression of clinical and histological signs of EAE after treatment with the β-adrenergic agonist isoproterenol (Chelmicka-Schorr et al., 1992).

Recently, de Keyser et al. (2004) discussed the role of astrocytic β2-adrenoceptors in multiple sclerosis (MS) disease progression. Correspondingly, therapies...
designed to elevate cAMP levels in astrocytes may have potential effects to prevent both relapse and progression of MS.

To investigate the putative immunoregulatory effects in the CNS of both 5-HT and NE, we examined the antidepressant venlafaxine, a highly selective 5-HT and NE reuptake inhibitor (SNRI) which blocks both 5-HT and NE transporters (SERT and NET) respectively.

We employed an inflammatory astroglia–microglia co-culture model (Faustmann et al., 2003, Hinkerohe et al., 2005) which allows mimicking of inflammatory conditions in an in-vitro bioassay. Especially, the activation of microglia and response of astroglia to microglial activation can be monitored in this assay. Since astrocytes seem to play a crucial role in the pathogenesis of inflammatory diseases of the CNS and represent pharmacological targets of antidepressants (Hertz et al., 2004) we studied the influence of exogenously applied venlafaxine on inflammatory markers including the degree of the astroglial coupling, the interleukin-6 (IL-6), IL-10, transforming growth factor-β (TGF-β) and IFN-γ response and the morphological change of microglial phenotype.

Methods

Cell culture

Primary cell cultures of glial cells were prepared from hemispheres of postnatal (P0–P2) Wistar rats according to Dermietzel et al. (1991) and Faustmann et al. (2003). Depending on the extent of shaking, the fraction of microglial cells remaining in the co-cultures varies between 5% (M5), comparable to the concentration found in healthy adult brain tissue, and 30% (M30) as determined by counting after fixation and immunohistochemical staining with the microglia marker ED1 (see Hinkerohe et al., 2005 for detailed methodology). The study was approved by the Bioethical Committee of the Ruhr-University Bochum, and experiments were performed in accordance with accepted guidelines for care and use of animals in research.

Administration of the antidepressant substance venlafaxine

Based on findings by Uhr and co-workers (2003), we incubated cells with 300 ng/ml to mimic cerebrum concentrations which are found after 1-wk treatment with the optimal dose of venlafaxine. In a further condition, we reduced the concentration by a factor of 10 (30 ng/ml) to create suboptimal dosing conditions. Drugs were dissolved in 50 μl phosphate buffered saline (PBS) and were added to M5 and M30 co-cultures for 16 h.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were quantified from cell culture supernatants by ELISA. Samples of supernatants were collected after incubation and stored at −20 °C until concentration determination. Quantikine-ELISA kits (R&D Sytstems, Minneapolis, MN, USA) were applied for quantification of rat IL-6, IL-10, IFN-γ and TGF-β according to the manufacturer’s protocol. Cytokine concentrations of all control (incubation with PBS for 16 h) and venlafaxine-treated (incubation for 16 h) samples were determined in duplicate. All data represent means of four independent experiments.

Functional coupling and membrane resting potential (MRP)

Astrogial MRP was determined by employing the whole-cell patch-clamp mode (see Hinkerohe et al., 2005 for detailed procedure). The technique allows simultaneous intracellular injection of Lucifer Yellow and monitoring of dye transfer to neighbouring cells. Numbers of coupled astroglial cells were counted 10 min after dye injection.

Immunofluorescence

Immunofluorescence was applied to co-cultures in order to assess the fraction of microglia within the astrocytic cultures. Briefly, we determined the density of astrocytes by immunolabelling the glial fibrillary acidic protein (GFAP) with a polyclonal antibody (1:100; Sigma G9269, Taufkirchen, Germany). Microglia were labelled by using a monoclonal antibody directed to the ED1 epitope (1:250; Serotec MCA 341R, Eching, Germany), which allowed classification of microglia as resting ramified (RRT), intermediate (INT) and activated, rounded phagocytic (RPT) phenotypes (Faustmann et al., 2003). For quantification of cell numbers immunocytochemically labelled cells were counter-stained with DAPI (4,6-diamidino-2-phenyl-indol) (1:2500; Sigma D9542) to visualize the nuclei.

Statistical analysis

Significance of differences between mean cytokine concentrations (IL-10 and TGF-β) were tested using the t test. Significant differences for the mean IL-6 and IFN-γ concentrations were determined with the Mann–Whitney U test.
Significant effects regarding the mean number of coupled astroglia were tested with the Mann–Whitney U test and differences between mean astroglial MRPs with the t test. Significant differences between mean fractions of active and resting microglia were tested with the t test. All statistical analyses were performed with SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

MRP of astrocytes and intercellular coupling

In M5 (5%) cultures incubated with PBS mean MRPs of $-69.56 \text{ mV (s.e.m.} = 2.46\text{)}$ which is in the range of the normal astrocytic MRP were detected (Figure 1). Increase of the microglia fraction to 30% caused a significant depolarization ($t = 4.94$, $p < 0.001$) of the MRP (mean $= -49.6 \text{ mV, s.e.m.} = 3.07\text{)}$ compared to M5. Incubation of 30 ng/ml venlafaxine with M30 prevented depolarization ($t = -2.28$, $p < 0.05$) of the membrane potential of astrocytes (mean $= -60.96 \text{ mV, s.e.m.} = 3.94\text{)}$ significantly. Venlafaxine 300 ng/ml revealed slightly lower but non-significant MRPs (mean $= -57.15\text{, s.e.m.} = 10.38\text{)}$.

For functional intercellular coupling (Figure 1), we found a significant reduction ($Z = -2.82$, $p < 0.01\text{)}$ of the number of coupled cells comparing M5 PBS incubated cultures (mean $= 13.18\text{, s.e.m.} = 3.36\text{)}$ with M30 cultures (mean $= 6.69\text{, s.e.m.} = 2.72\text{)}$. The incubation of M30 with 300 ng/ml venlafaxine could almost restore ($Z = -2.05$, $p < 0.05\text{)}$ the number of coupled cells (mean $= 10\text{, s.e.m.} = 1.08\text{)}$.

Cytokine concentrations

M5 cultures incubated with PBS revealed a significantly lower concentration of pro-inflammatory (IL-6, IFN-$\gamma$) cytokines and a higher concentration of anti-inflammatory cytokines (IL-10, TGF-$\beta$) compared to the M30 cultures (Figure 2). Markedly, the incubation of M30 cultures with venlafaxine significantly altered cytokine concentrations dose-dependently through elevation of TGF-$\beta$ and suppression of IL-6 and IFN-$\gamma$ secretion. The incubation of 30 ng/ml venlafaxine significantly ($Z = -2.94$, $p < 0.01\text{)}$ elicited a decrease of the mean IL-6 concentration from 185.71 pg/ml (s.e.m. = 31.56) to 116.6 pg/ml (s.e.m. = 11.58), whereas the effect was not significant for 300 ng/ml. By incubating M30 cultures with 30 ng/ml venlafaxine we found significantly higher ($t = -2.19$, $p < 0.05\text{)}$ mean TGF-$\beta$ concentrations under inflammatory conditions (M30 PBS = 14.46 pg/ml, s.e.m. = 3.18) compared to M30 venlafaxine-treated cultures (30 ng/ml = 23.1 pg/ml, s.e.m. = 1.87). For IFN-$\gamma$, the incubation with 300 ng/ml venlafaxine could reduce

![Figure 1.](image-url)
mean concentration from 77.3 pg/ml (S.E.M. = 4.17) for M30 PBS to 67 pg/ml (S.E.M. = 4.5).

For IL-10 concentrations, we did not find significant effects for venlafaxine; M5 PBS incubated cultures revealed significantly higher mean concentrations than all other conditions.

Change in microglial phenotype

While the RRT dominates in cultures with a low number of microglia (mean M5 = 37.6%, S.E.M. = 7.44; mean M30 = 11.73%, S.E.M. = 2.87; t = 3.166, p < 0.01) (Figure 3), the activated RPT dominates in cultures of M30. In M5 cultures, 29.07% (S.E.M. = 4.76) of the cells were activated whereas 61.58% (S.E.M. = 3.28) RPTs were found in M30 (t = 5.551, p < 0.001). The incubation of venlafaxine with M30 co-cultures for 16 h resulted in a profound deactivation of microglia, comparable to the conditions observed in M5 conditions. When M30 co-cultures were treated with 30 ng/ml venlafaxine a ratio of 43.55% (S.E.M. = 8.07) RRT microglial cells and 34.80% (S.E.M. = 4.34) activated RPTs was determined. The differences reached high significance (RRT: t = -4.59, p < 0.001; RPT: t = 4.80, p < 0.001). The addition of 300 ng/ml venlafaxine could also restore the activation pattern found in M5 cultures. Under incubation only 20.40% (S.E.M. = 2.23) of microglia were active (t = 9.65, p < 0.001) whereas 47.18% (S.E.M. = 4.16) were RRT (t = -7.25, p < 0.001).

Discussion

Our results for M5 and M30 co-cultures are in good accord with previous findings described by Faustmann et al. (2003) and Hinkerohe et al. (2005). They recently provided evidence that an increase of microglia cells to about 30% elicits a significant activation compared to cultures obtained with 5% microglia. This phenotypic activation was accompanied by a decrease of astroglial MRP and reduced intercellular coupling indicating a clear-cut correlation between microglia fraction and basic astrocytic properties.

In this investigation, we yielded stringent evidence that the SNRI venlafaxine reversed the inflammatory conditions of M30 cultures in a dose-dependent fashion. Incubation of M30 cultures with venlafaxine was capable of preventing microglial activation, strengthening the astroglial coupling and minimizing pro-inflammatory cytokine secretion. Markedly, the lower dose (30 ng/ml) was more effective on changes of astrocytic properties compared to the higher dose application. Incubation with the higher dose (300 ng/ml) equals a treatment in humans of >150 mg/d when considering plasma and cerebrum levels (Uhr et al., 2003) of venlafaxine-treated mice. When assuming a linear dose–serum concentration dependency (Reis et al., 2002), a human dose of 15 mg/d can be approximated for the lower concentration used in the present study.
These results correlate with some previous findings on the neuroprotective effects of venlafaxine (Xu et al., 2003). Those authors described venlafaxine’s effects on rats’ hippocampal BDNF and reported an increase in BDNF-positive pyramidal neurons after chronic treatment with 5 mg/kg.d. The higher dose (10 mg/kg) decreased the intensity of BDNF immunostaining in all subareas.

Present results strongly promote the concept of anti-inflammatory properties of venlafaxine in vitro and may put a new complexion on the relationship between neuroinflammatory and other pathogenic CNS processes. Markedly, the substance exerts effects on astrocytes which recently emerged as potential targets for inflammatory CNS diseases (Ransom et al., 2003). Monoamine transporters (Inazu et al., 2003) as well as adrenergic receptors (Hertz et al., 2004) which have been identified on astrocytes might play a key role in mediating anti-inflammatory effects by anti-depressants.

Figure 3. Change of microglial phenotype from activated to resting morphology by incubation with venlafaxine. Each bar (a) represents the mean percentage ± S.E.M. of resting (□), intermediate (■) or active (□) microglial cells in the co-culture after 16 h of incubation with indicated substance concentration or vehicle. Data are from at least four different experiments. Significant differences are indicated (** p < 0.01, *** p < 0.001). The left image (b) displays astrocytes (green) and mainly resting ramified microglial cells (red, indicated by a star) whereas microglial cells in the right image largely constitute the round phagocytic phenotype (both ×63 magnification).
Cytokines which have been found to play a key role in the pathogenesis of inflammatory CNS diseases were greatly impacted by venlafaxine. For instance, IL-6 which is released in the CNS during various pathological conditions, including Alzheimer’s disease, MS, CNS trauma, and viral and bacterial meningitis (Gruol and Nelson, 1997) was reduced by venlafaxine. Moreover, IFN-γ which was significantly lowered by venlafaxine is a suspected participant in the pathogenesis of MS (Becher et al., 1999). The augmentation of the anti-inflammatory cytokine TGF-β by venlafaxine strengthens the immunoregulatory effects since TGF-β was shown in rodents to prevent the development and/or exacerbation of disease symptoms in EAE (Johns et al., 1991). Besides the neuroimmunological involvement of cytokines, considerable clinical and experimental data support the existence of a relationship between cytokines and depression (Levine et al., 1999).

Interestingly, the well-established tricyclic antidepressant amitriptyline was recently found to inhibit the secretion of pro-inflammatory cytokines in rat mixed glial and microglial cell cultures (Obuchowitz et al., 2006). Similarly to venlafaxine, amitriptyline inhibits 5-HT and NE reuptake but acts in a more unspecific way, supporting the thesis of a noradrenergic- and serotoninergic-mediated immunoregulation.

In conclusion, antidepressants targeting 5-HT and NE transporters seem to suppress inflammatory processes in vitro. Further studies in animal models (e.g. EAE) must be conducted to demonstrate antidepressants as a putative treatment option for inflammatory CNS pathologies.

Acknowledgements

The authors thank Sabine Schreiber-Minjoli, Piotr Chartowski and Michael Schiff for excellent technical assistance. They also thank Sean Patrick Morrissey for comments on this project. Venlafaxine was kindly provided by Wyeth Pharma.

State of Interest

None.

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