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Functional CXCR4 receptor development parallels sensitivity to HIV-1 gp120 in cultured rat astroglial cells but not in cultured rat cortical neurons

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The α chemokine receptor CXCR4 is used as the major coreceptor for the cell entry of T-cell-tropic human immunodeficiency virus-1 (HIV-1) isolates. Activation of this coreceptor by its natural ligand SDF1α is associated with an intracellular Ca^{2+} increase. Because the HIV-1 glycoprotein 120 (gp120) is shedded from the surface of HIV-1–infected cells and is regarded as an injurious molecule in the pathogenesis of HIV-1–associated encephalopathy (HIVE), we investigated the effects of gp120 on the intracellular Ca^{2+} regulation of astrocytes and neurons. After 5 days *in vitro* (DIV), SDF1α (50 nM) elicited a pertussis toxin–sensitive intracellular Ca^{2+} increase due to Ca^{2+} release from internal stores that was reduced by a blocking monoclonal antibody against the CXCR4 receptor in astrocytes and neurons. Parallel with the development of the SDF1α response, cells became sensitive to direct application of gp120 (1.25 μg/ml), which, similarly to SDF1α, elicited a transient intracellular Ca^{2+} increase. However, short-term incubation with gp120 for 60 to 120 min induced a reduction of glutamate- or ATP-evoked intracellular Ca^{2+} responses only in astrocytes and not in neurons, although functional CXCR4 receptors were expressed in both cell types. Therefore, our data strongly suggest that the CXCR4 receptor–mediated intracellular signaling pathway of gp120 differs in astrocytes and neurons. *Journal of NeuroVirology* (2002) **8,** 411–419.

Keywords: cell culture; chemokine receptor; glutamate; HIV-1–associated encephalopathy; intracellular calcium regulation

Introduction

Human immunodeficiency virus-1 (HIV-1)–associated encephalopathy (HIVE) is a severe neurological complication of HIV-1 infection in about one third of adults and half of the infected children, leading to motor and cognitive deficits up to overt dementia (McArthur *et al*, 1993; Berger and Arendt, 2000). Indirect mechanisms due to soluble factors that are locally released seem to play a crucial role in the pathogenesis of HIVE (Epstein and Gendelman, 1993; Kaul *et al*, 2001). This hypothesis is supported by observations that such factors can be specifically detected within brains of HIVE patients in postmortem examination, and that they exert neurotoxic and pathophysiological effects in cell culture (Nath and Geiger, 1998; Nath *et al*, 2000). The hypothesis gained further evidence by our recent findings that cerebrospinal fluid (CSF) from HIVE patients contains molecular factors that interfere with intracellular Ca^{2+} regulation in astrocytes, whereas CSF samples from HIV-1–seropositive patients without encephalopathy do

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not induce a Ca^{2+} dysregulation in glial cells (Köller *et al*, 2001b).

Among the viral soluble factors that might contribute to the pathogenesis of HIVE, HIV-1 gp120 is of special interest, because it interacts with astroglial and neuronal function by activating chemokine receptors that are used as coreceptors by HIV-1 during cell entry (Hesselgesser *et al*, 1997, 1998; Gabuzda and Wang, 2000). Activation of chemokine receptors induces an intracellular Ca^{2+} increase that is mediated by Ca^{2+} release from internal stores. The α chemokine receptor CXCR4 has been found on astroglial and neuronal cells (Bajetto *et al*, 1999; Klein *et al*, 1999). Gp120 from T-cell-tropic or dual-tropic HIV-1 isolates induces an intracellular Ca^{2+} response after binding at this receptor (Meucci *et al*, 1998). Very little, however, is known about the functional consequences of prolonged CXCR4 receptor activation, which could appear within the brains of HIV-1–infected patients, for example, by local release of gp120 from infected microglial cells. Therefore, we studied the effects of direct application and short-term incubation of gp120 on intracellular Ca^{2+} regulation of cultured astrocytes and neurons. Moreover, we used a human neuroteratocarcinoma cell line (NT2 cells) as a human-derived culture model with neuronal characteristics. Surprisingly, we did not find a general correlation between the presence of a functional CXCR4 receptor, measured by recording of a SDF1α-evoked intracellular Ca^{2+} increase, and the gp120-induced intracellular Ca^{2+} dysregulation, measured by a Ca^{2+} increase upon glutamate or ATP application.

Results

Development of functional CXCR4 receptor in astrocytes and neurons

HIV-1 envelope protein gp120 is one of the viral proteins that contribute to the pathogenesis of HIV-1–associated encephalopathy. Infected cells in the neighbourhood of astrocytes and neurons, especially macrophages and microglia cells, are known to release gp120 into the microenvironment. Thus, we were interested in analyzing acute (by direct application) and subacute (by incubation for 2 h) functional effects of gp120 on cortical astrocytes and neurons. Gp120 from T-cell-tropic virus strains activates the α chemokine receptor CXCR4, and, therefore, we analyzed the functional development of the CXCR4 receptor using bath application of its natural ligand SDF1α in cultured astrocytes and neurons (Figure 1).

In the presence of serum, rat cortical astrocytes did not show a Ca^{2+} response upon SDF1α application during a 2-week culture period, indicating that CXCR4 is not functionally active in proliferating astrocytes (Figure 2). To reduce proliferation and induce partial differentiation, we prolonged cultivation at higher cell density in serum-free medium.

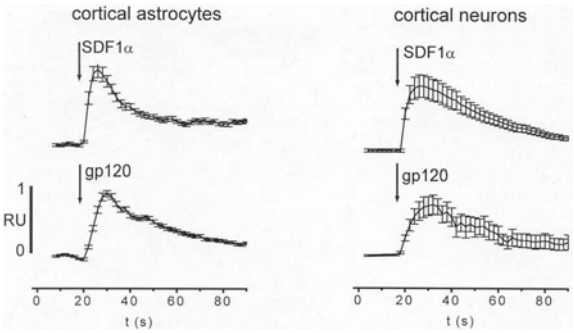

Figure 1 Bath application of SDF1α and gp120 elicited an increase of intracellular Ca^{2+} in astrocytes and neurons. Intracellular Ca^{2+} concentrations were recorded in Fura-2–loaded cultured cortical astrocytes and neurons. In astrocytes grown in serum-free culture medium, direct application of SDF1α (50 nM, see *arrows*) and gp120 (1.25 μg/ml, see *arrows*) elicited a transient increase of intracellular Ca^{2+} concentration, whereas proliferating astrocytes grown in serum-containing culture medium were insensitive to both SDF1α and gp120. The figure shows the intracellular Ca^{2+} increase in an astroglial culture 10 days *in vitro* (DIV) and with serum removal from day 5 on (*left*). Cultured neurons respond similarly to SDF1α and gp120, data are shown from neurons 7 DIV (*right*). The data are given as means ± SEM of the intracellular Ca^{2+} concentrations of 8 to 12 cells recorded simultaneously in the same culture dish. RU, ratio units.

In contrast to the nonconfluent astrocytes proliferating in serum-containing culture medium, astroglial cells in confluent monolayers regularly responded to SDF1α (50 nM) application with a transient intracellular Ca^{2+} increase 2 days after serum removal (Figure 2). The Ca^{2+} response could be demonstrated in all intact cells tested between day 2 after serum removal and day 12 (Figure 2). These results show that partial differentiation following serum removal is necessary for the detection of a functional CXCR4 receptor in cultured astrocytes.

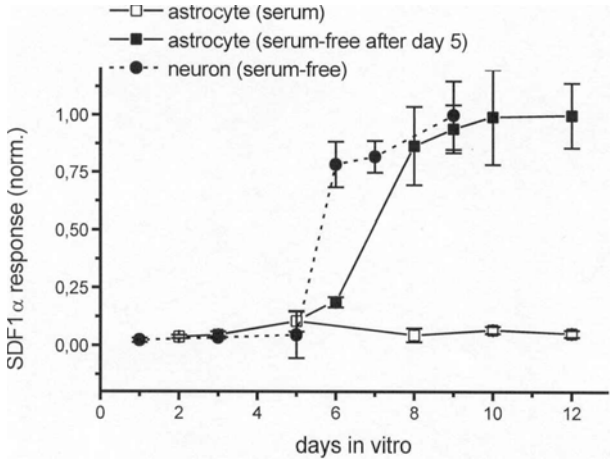

Figure 2 Development of SDF1α-elicited intracellular Ca^{2+} increase in astrocytes and neurons. Intracellular Ca^{2+} responses upon SDF1α application were regularly observed in astroglial cells 2 days after serum removal (■), whereas in astrocytes continuously grown in serum containing culture medium SDF1α application did not evoke an intracellular Ca^{2+} increase (□). Cortical neurons were sensitive to SDF1α after 5 days *in vitro* (●).

To analyze whether the CXCR4 receptor was similarly expressed in neurons, we applied SDF1α to neuronal cultures that were grown in astrocyte conditioned serum-free medium. Although cortical neurons failed to show an intracellular Ca^{2+} response upon SDF1α application during the first 4 days *in vitro* (DIV), SDF1α induced a transient intracellular Ca^{2+} increase in cells from 5 to 9 DIV (Figure 1). Similar to astrocytes, CXCR4 receptor was expressed in neurons within 1 week in culture (Figure 2).

The findings in astrocytes and neurons suggest that expression of the functional CXCR4 receptor is dependent on at least partial differentiation of the cells. Therefore, we chose human neuroteratoma (NT2) cells as a model system in which neuronal differentiation can be induced to study functional CXCR4 receptor development in precursor and neuronally differentiated NT2 cells. Although SDF1α did not induce an intracellular Ca^{2+} increase in undifferentiated precursor cells, neuronally differentiated NT2 cells responded with a Ca^{2+} increase upon SDF1α application. SDF1α response was observed from 5 to 18 DIV in neuronally differentiated cells (data not shown). Thus, we have shown that astrocytes and neurons in serum-free medium and neuronally differentiated NT2 cells express functional CXCR4 receptors in culture.

Pharmacological properties of the CXCR4 receptor–mediated Ca $^{2+}$ response

In order to characterize pharmacological properties of the CXCR4 receptor in astrocytes and neuronal cells under our experimental conditions, we tested the SDF1α-induced intracellular Ca^{2+} response for its sensitivity to thapsigargin (Tg), which depletes intracellular Ca^{2+} stores and pertussis toxin (PTX), which uncouples the receptor from its respective G-proteins (Klein *et al*, 1999). As representatively shown for the recordings in astrocytes (Figure 3), the intracellular Ca^{2+} increase was unaffected by removal of extracellular Ca^{2+}, but sensitive to Tg, indicating that the observed intracellular Ca^{2+} increase was mainly due to Ca^{2+} release from internal stores (Figure 3). Similarly, as reported by Klein *et al* (1999), the CXCR4 receptor–induced Ca^{2+} response was sensitive to PTX preincubation, indicating that the intracellular signaling was mediated by PTX-sensitive G-proteins. The effects were specific for the CXCR4 receptor, because incubation of cells with the blocking monoclonal antibody against the CXCR4 receptor (12G5) led to a significant reduction of the Ca^{2+} response upon SDF1α application (Figure 3) whereas incubation with a monoclonal antibody against the β chemokine receptor CCR5 did not reduce the SDF1α responses.

CXCR4 receptor–mediated intracellular Ca^{2+} increase induced by gp120

The recordings of the pharmacological properties of the CXCR4 receptor revealed that it was functionally

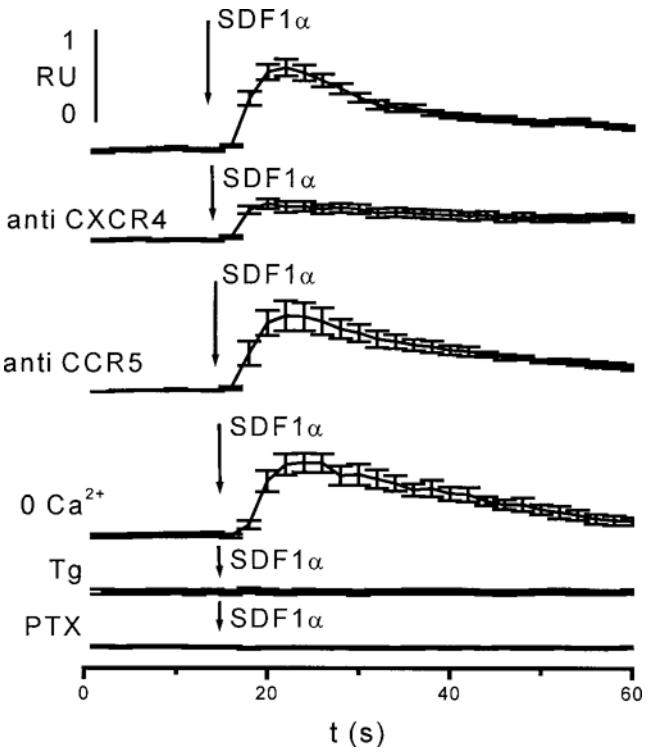

Figure 3 Pharmacological characterization of SDF1α responses. SDF1α (50 nM) evoked an intracellular Ca^{2+} increase (RU = ratio units) that was reduced by blocking of the α chemokine receptor CXCR4 by a blocking monoclonal antibody (antiCXCR4, 12G5, 10 μg/ml) but not by an anti-CCR5 antibody (10 μg/ml). Removal of extracellular Ca^{2+} (0 Ca^{2+}) did not reduce the SDF1α-evoked Ca^{2+} response, whereas the Ca^{2+} response was sensitive to thapsigargin (Tg; 20 μM, 30 min prior to SDF1α application) and pertussis toxin (PTX; 250 ng/ml, 1 h prior to SDF1α application). Typical recordings are shown from experiments on astrocytes. Similar results were obtained from neurons.

active in differentiated astrocytes and neuronal cells. To address the question of whether gp120 elicited a similar Ca^{2+} response, we directly applied recombinant gp120 from HIV-1 strain IIIB to the differentiated cells expressing functional CXCR4 receptor. The acute Ca^{2+} response elicited by gp120 (1.25 μg/ml) application was undistinguishable from the effect of SDF1α (Figure 1). Preincubation of cells with the CXCR4 receptor–blocking monoclonal antibody (12G5) reduced the gp120 response, indicating that it was also mediated by CXCR4 receptor activation (Figure 4). This is further supported by our observation that cells lacking functional CXCR4 receptors, that is, undifferentiated astrocytes and neuronal cells during day 1 to 3 in culture, did not respond to gp120 application with an intracellular Ca^{2+} increase (data not shown). Likewise, the gp120-induced intracellular Ca^{2+} increase was abolished by Tg and PTX, showing the same pharmacological properties as the SDF1α response (data not shown).

In order to test the effects of gp120 locally released from glycoprotein-expressing cells, which may reflect the situation in the HIV-1–infected brain

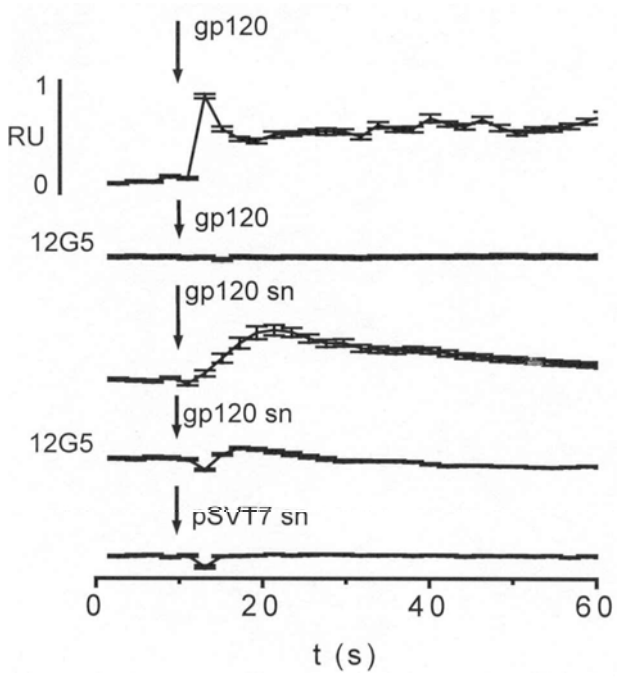

Figure 4 HIV-1 gp120 evoked intracellular Ca^{2+} increase. Recombinant gp120 (1.25 μg/ml) elicited an intracellular Ca^{2+} increase in astrocytes similar to SDF1α that was nearly completely reduced after preincubation of cells with a blocking antibody against the CXCR4 receptor (12G5; 10 μg/ml). Supernatant from HeLa-T4$^+$ cells secreting gp120 (gp120sn) also induced an intracellular Ca^{2+} increase that was similarly reduced by 12G5. Supernatant from HeLa-T4$^+$ cells transfected with the parental vector without coding for gp120 (pSVT7sn) served as control and did not elicit a Ca^{2+} response.

even more authentically, we also tested whether the supernatant from a transiently transfected cell line secreting gp120 also induced a Ca^{2+} response. As expected, we found an intracellular Ca^{2+} increase upon application of supernatant from gp120-secreting cells, whereas supernatant from cells that were transfected with only the parental cloning vector did not affect the intracellular Ca^{2+} concentration (Figure 4). Similar to the results using SDF1α or recombinant gp120 application, the Ca^{2+} response to application of the gp120-containing supernatant was reduced in cultures pretreated with the neutralizing antibody against the CXCR4 receptor (Figure 4) and abolished by Tg and PTX, which again resembled the pharmacological properties of the SDF1α response (data not shown). Thus, we showed that supernatant from gp120-expressing cells was effective in inducing the CXCR4 receptor–mediated Ca^{2+} increase, similar to SDF1α and recombinant gp120, suggesting that gp120 released from HIV-1–infected cells is able to modulate intracellular Ca^{2+} regulation in neighboring astrocytes and neurons.

Effects of prolonged application of gp120

In the pathogenesis of HIVE, the infected cells can shed gp120 during the whole period of active gene expression, suggesting that astrocytes and neurons are chronically exposed to gp120. For this reason, we were interested in functional effects of prolonged application of gp120 on astrocytes and neurons within the first 2 h of gp120 exposure. We asked whether prolonged application of gp120 induces a sustained intracellular Ca^{2+} increase, with a shift of the Ca^{2+} concentration baseline, which often represents an initial step in cell damage in various cell injury models. Therefore, we incubated astrocytes and neurons for 1 h in culture medium containing recombinant gp120 (1.25 μg/ml), or alternatively, in supernatants from cells expressing and secreting gp120. Under these experimental conditions, baseline intracellular Ca^{2+} concentrations were found unchanged, which was in line with the observation that the SDF1α- and the gp120-induced intracellular Ca^{2+} increase was transient and returned to baseline levels within 1 or 2 min.

Although baseline intracellular Ca^{2+} concentrations were undisturbed by gp120, we now asked whether the intracellular Ca^{2+} regulation may be affected, which could impair the capability of astrocytes and neurons to respond to physiological stimuli known to increase intracellular Ca^{2+} concentrations, such as glutamate or ATP.

In astrocytes, both glutamate (1 mM) as well as ATP (1 mM) induced an intracellular Ca^{2+} increase and Ca^{2+} responses were present in cells irrespective of gp120 preincubation. However, the response to glutamate application was significantly reduced in cells preincubated with gp120 compared to controls (Figure 5**A**). Gp120 did not seem to interact directly with the glutamate receptor, because the glutamate response in the presence of gp120 (or SDF1α) without preincubation was not reduced, whereas an incubation period of 30 min or longer was necessary for the gp120-induced reduction of the glutamate response (data not shown).

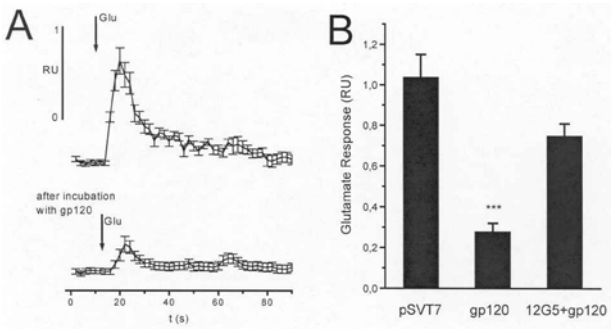

Figure 5 HIV-1 gp120 reduced the glutamate-evoked Ca^{2+} response in astrocytes. Glutamate (1 mM) elicited a Ca^{2+} response in astrocytes that was significantly reduced by gp120 incubation (for 60 min, starting 120 min prior to Ca^{2+} recordings) of cells 8 to 12 days *in vitro* and grown in serum-free medium (**A**). The gp120 effect was reduced in experiments in which the CXCR4 receptor was blocked by the monoclonal antibody against the receptor (12G5) (**B**). Data are shown as means and standard errors of the means. ***Statistically significant differences with $P < .001$. RU, ratio units.

Similar to the glutamate response, the ATP-induced intracellular Ca^{2+} increase was also significantly diminished in gp120-preincubated astrocytes: 1.51 ± 0.20 ratio units in gp120-preincubated cells ($n = 36$ cells, $P < .001$) compared to 2.90 ± 0.21 ratio units in cells ($n = 38$) not incubated with gp120. This shows that prolonged application of gp120 induced a reduction of physiological Ca^{2+} responses irrespective of the stimulus tested.

The gp120-induced reduction of the Ca^{2+} responses was only observed in cells 8 DIV and longer, which were grown in serum-free medium were sensitive to SDF1α. In cells from sibling cultures from the same preparation, which were cultured in the presence of serum and were insensitive to SDF1α, gp120 did not induce a reduction of Ca^{2+} response. This suggests that the gp120-induced reduction of glutamate or ATP response was mediated by functional CXCR4 receptors. This hypothesis was further substantiated by the observation that blockade of the CXCR4 receptor by the blocking antibody against the CXCR4 receptor significant reduced the effect of gp120 preincubation on the glutamate response (Figure 5**B**). We then asked whether incubation of astrocytes with the natural ligand of the CXCR4 receptor, SDF1α, also induced a reduction of glutamate responses, which was found to be the case. Glutamate induced a significant reduction in intracellular Ca^{2+} increase in cells preincubated with SDF1α (Table 1). This further underlines the key role of the CXCR4 receptor in the gp120- (and SDF1α-) induced intracellular Ca^{2+} dysregulation.

In cortical neurons, however, the findings were clearly different. Similar to astrocytes, SDF1α and gp120 responses were detectable in cells older than 5 DIV. Unexpectedly, preincubation with gp120 (Figure 6) or SDF1α (Table 1) did not induce a reduction of the intracellular Ca^{2+} increase upon glutamate application in cells expressing functional CXCR4 receptor. Interestingly, neuronally differentiated NT2 cells were sensitive to prolonged gp120 application (Figure 6). In this case, ATP was used as a standard stimulus because precursor cells did not respond to glutamate application with an intracellular Ca^{2+} increase similar to other tumor cells (Weydt *et al*, 1997). Incubation with gp120 induced a reduction of the ATP response only in differentiated NT2 cells but not in SDF1α-insensitive precursor cells.

Table 1 Incubation with SDF1α induces a reduction of glutamate response in cultured astrocytes but not in neurons

	CTRL (ratio units)	*Incubation with SDF1α (ratio units)*	
Astrocytes	1.04 ± 0.11 ($n = 34$)	0.49 ± 0.14 ($n = 46$)	$p < .001$
Neurons	1.02 ± 0.08 ($n = 114$)	1.10 ± 0.10 ($n = 125$)	n.s.

Note. Cells were incubated with SDF1α for 60 min and intracellular Ca^{2+} responses upon glutamate (1 mM) application were recorded 120 min after beginning of incubation. Although SDF1α induced a reduction of glutamate responses in astrocytes, glutamate responses of cultured neurons were unaffected by SDF1α incubation. CTRL, control recordings without SDF1α incubation. n.s. = not significant.

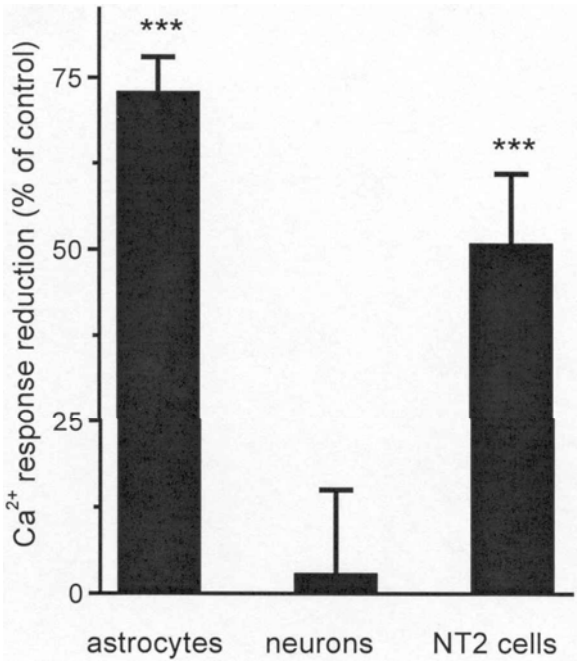

Figure 6 Incubation with HIV-1 gp120 induced a reduction of calcium responses in SDF1α-sensitive astrocytes and NT2 cells but not in neurons. Cells were incubated for 1 h in culture medium containing gp120 (1.25 μg/ml) or in supernatants from gp120-secreting HeLaT4$^+$ cells and the Ca^{2+} response upon glutamate (astrocytes and neurons) or ATP (NT2 cells) application was measured 2 h after the beginning of gp120 incubation. Incubation with gp120 induced a significant ($^{***}p < .001$) reduction of the glutamate response in astrocytes and NT2 cells expressing functional active CXCR4 receptors as shown by its sensitivity to SDF1α. Interestingly, cortical neurons did not show a reduction of the glutamate response, although SDF1α regularly elicited a Ca^{2+} response.

The findings in astrocytes and differentiated NT2 cells show that the expression of functional CXCR4 receptor is a prerequisite for the gp120-induced dysregulation of Ca^{2+} responses. The insensitivity of neurons in this respect, however, reveals that the CXCR4 receptor–mediated gp120-induced intracellular Ca^{2+} increase is not sufficient for the intracellular Ca^{2+} dysregulation upon prolonged gp120 application. This suggests that intracellular signaling pathways apart from or beyond Ca^{2+} release from intracellular stores are specifically present in astrocytes and differentiated NT2 cells, but absent or ineffective in neurons.

Discussion

HIV-1 gp120 led to an intracellular Ca^{2+} increase in neurons mediated by several mechanisms, including

facilitation of N-methyl-D-aspartate (NMDA)- and glutamate-evoked Ca^{2+} increase (Lo *et al*, 1992; Holden *et al*, 1999), Ca^{2+} mobilization from intracellular stores (Medina *et al*, 1999), and activation of the CXCR4 receptor similar to receptor activation by SDF1α (Meucci *et al*, 1998). In accordance with the reports of Meucci and coworkers (1998) on neurons and of Liu and coworkers (2000) on macrophages, our data show that gp120 from a T-cell-tropic virus strain activates the α chemokine receptor CXCR4 and induces an intracellular Ca^{2+} increase in cultured astrocytes and neurons following induction of differentiation. Our finding that astrocytes, neurons, and NT2 cells fail to respond to SDF1α and gp120 unless they are induced to differentiate suggest that cellular differentiation is a prerequisite for the expression of functional CXCR4 receptor. However, most likely, the cells can be still immature. This is supported by the observations that in the neonatal mouse cerebellum, the CXCR4 message peaks during the first postnatal week when postmitotic but still immature granule cells have started their migration to form the internal granule cell layer (Klein *et al*, 2001).

The functional response of the CXCR4 receptor activation, that is, the SDF1α- and gp120-evoked intracellular Ca^{2+} response and gp120-induced reduction of glutamate or ATP could be reduced by blocking the CXCR4 receptor using the monoclonal antibody 12G5, emphasizing the specificity for this gp120 signaling pathway. The same antibody has been shown previously to block glial and neuronal apoptosis, which could be induced with gp120 from blood-derived T-cell-tropic virus isolates (Hesselgesser *et al*, 1997; Meucci *et al*, 1998; Kaul and Lipton, 1999; Ohagen *et al*, 1999) but not with HIV-1 envelope proteins from brain-derived macrophage tropic virus strains (Ohagen *et al*, 1999).

Incubation of cells with PTX, which blocks the SDF1α response, revealed a G-protein–coupled intracellular signaling pathway. The functional results obtained in our culture systems therefore closely resemble, in this respect, previous findings on the expression and function of the CXCR4 receptor on cultured astrocytes and neurons (Klein *et al*, 1999). However, through measurement of intracellular Ca^{2+} transients upon glutamate and ATP application, we found reduced Ca^{2+} responses only in SDF1α-sensitive astrocytes and differentiated NT2 cells incubated with gp120. Although cortical neurons similarly responded to direct SDF1α and gp120 application with an intracellular Ca^{2+} increase, gp120 incubation did not induce a reduction of the glutamate response. This suggests that direct activation of the CXCR4 receptor shares similar intracellular signaling pathways in astrocytes and neurons with an intracellular Ca^{2+} release from internal stores. However, cell-specific intracellular processes lead to a disturbance of intracellular glutamate and ATP responses only in astrocytes after prolonged CXCR4 receptor activation. Another example of cell-specific CXCR4 receptor signaling is the observation that gp120 activates mitogen-activated protein (MAP) kinases, mediated by the CXCR4 receptor, only in neurons but not in astrocytes (Lazarini *et al*, 2000).

The intracellular mechanisms that lead to a reduction of glutamate- and ATP-induced intracellular Ca^{2+} increase in astrocytes and of ATP-induced Ca^{2+} increase in NT2 cells are so far unclear. Very recently, Bezzi *et al* (2001) reported an astroglial glutamate release induced by CXCR4 receptor activation in the presence of tumor necrosis factor alpha (TNFα), which could induce a glutamate receptor desensitization followed by reduced glutamate responses. However, cells were transferred from incubation solution containing SDF1α or gp120, and eventually released glutamate, to fresh saline for recording. Additionally, the glutamate release depended on the presence of TNFα, which was released by microglia in slice experiments. In our astroglial cultures, efforts were made to markedly reduce the number of contaminating microglia and in the NT2 cultures microglia was absent. The observation that ATP-evoked responses were also reduced argues against a desensitization of glutamate receptors as underlying mechanism.

A direct interaction between the gp120 and glutamate or ATP receptors is very unlikely because (i) the glutamate response elicited in the presence of gp120 without incubation were unchanged; (ii) in neurons that bear similar glutamate receptors as astrocytes, gp120 did not reduce the glutamate response; and (iii) it is unlikely that a single receptor-blocking compound affects glutamate as well as ATP receptors. Activation of CXCR4 receptors seems to be necessary for this gp120 effect on glutamate responses because blocking of the receptor diminished the reduction of the glutamate response. Activation of the CXCR4 receptor by incubation with SDF1α also reduced the glutamate-evoked intracellular Ca^{2+} increase, which further emphasizes that CXCR4 receptors were involved. Application of gp120 resulted in an increase in intracellular Ca^{2+} concentration that returned to the baseline within several minutes. Because the glutamate application was performed 120 min after gp120 application, a prolonged Ca^{2+} increase with the consequence of a reduced glutamate-evoked Ca^{2+} increase could be excluded. As expected, the intracellular Ca^{2+} concentration was not different in cells incubated with gp120 and controls. Therefore, our data support the hypothesis of Lazarini and coworkers (2000) that gp120 activates more complex intracellular signaling pathways than the G-protein–coupled Ca^{2+} release from intracellular stores. In this respect, the reduction of glutamate responses by gp120 in astrocytes, but not in neurons, suggest a specific vulnerability of astroglial cells.

The difference in the gp120-induced reduction of glutamate response in neurons and astrocytes may also reflect different functional roles of these cells during inflammatory processes. Astrocytes, for example, respond to stimulation by interferon γ by

up-regulation of major histocompatibility complex (MHC) class I molecules and β_2 microglobulin. This response, however, was not found in bioelectrically active, healthy neurons. After blocking neuronal sodium-dependent action potentials by tetrodotoxin, however, neurons became sensitive to interferon γ (Neumann *et al*, 1997). The authors concluded that bioelectrically active neurons are able to reduce their responses to inflammatory stimuli, with the aim to reduce immunological surveillance. Our work also showed differences in the response of neurons and astrocytes to incubation with the cytokine TNFα, which is also regarded as an injurious molecule in HIVE (Epstein and Gendelman, 1993). Incubation of astrocytes with TNFα led to a slow intracellular Ca^{2+} increase and a reduction of inwardly rectifying K^+ (K_{IR}) currents specifically in astrocytes, but not in neurons (Köller *et al*, 1996b, 1998). Secondary to the cell depolarization due to the decrease of K_{IR} currents, the glutamate-induced intracellular Ca^{2+} increase was reduced in TNFα-incubated astrocytes, which was also not found in neurons (Köller *et al*, 2001a). Being at least partially immunocompetent cells, astrocytes seem to be sensitive to inflammatory stimuli also with respect to their electrophysiological porperties. Due to the tight coupling of neuronal and astroglial function (Nedergaard, 1994; Parpura *et al*, 1994), disturbance of astroglial Ca^{2+} regulation would have great impact on the function of the neuronal network. Our previous findings on TNFα and here on gp120 suggest that astrocytes are specifically vulnerable to inflammatory injuries and astroglial dysfunction may contribute to the pathogenesis of neurological symptoms in HIVE.

Materials and methods

Materials
Recombinant HIV-1 IIIB gp120 was a generous gift from ImmunoDiagnostics and the NIBSC Centralised Facility for AIDS Reagents (Potters Bar, Hertfordshire, UK). Recombinant human SDF1α, the anti-CXCR4 monoclonal antibody (12G5), and the anti-CCR5 monoclonal antibody were purchased from R&D Systems (Wiesbaden, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) unless otherwise indicated.

Cell cultures
Glial cells were prepared from hemispheres of newborn Wistar rats and grown in media containing 10% fetal calf serum as reported previously (Köller *et al*, 1998, 2001a). After 10 days, cells were shaken vigorously for 12 to 16 h on a rotary platform and redissociated to remove contaminating oligodendrocytes and neurons. Cells were plated on glass coverslips coated with poly-D-lysine (0.1 mg/ml, 24 h, 4°C). After a confluent monolayer had been established again, cells were transferred to serum-free culture medium consisting of a 3:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's F12 nutrient mixture supplemented with 5 μg/ml insulin, 100 μg/ml transferrin, 5.2 ng/ml sodium selenite, 16 μg/ml putrescine, and 7 ng/ml progesterone. Astrocytes were identified by immunohistochemical staining for glial fibrillary acidic protein (GFAP) and were stained positive by more than 95% (Stichel and Müller, 1992). Cells were used for experiments 1 to 3 weeks after transfer to serum-free medium.

Cortical neurons were prepared from hemispheres from embryonic Wistar rats at embryonic day 15 and grown in serum-free astrocyte-conditioned culture medium as described previously (Köller *et al*, 1996a). Cells were plated on glass coverslips coated with poly-D-lysine (0.1 mg/ml, 24 h, 4°C) and laminin (13 μg/ml, 24 h, 4°C). Culture medium consisted of the serum-free medium given above, which was preconditioned by a spatially separated monolayer of astrocytes for at least 3 days. Ca^{2+} measurements were performed after 7 to 12 days of culture.

Human embryonal carcinoma cell line NT2
A human embryonal carcinoma cell line NT2 (Neelands *et al*, 1999) was used to study receptor-operated intracellular Ca^{2+} changes as described previously (Köller *et al*, 2001c). The cells differentiate from a committed neuronal precursor stage to postmitotic neurons, depending on the culture conditions used. In this study, we used cells that were grown on glass coverslips coated with poly-D-lysine (0.1 mg/ml, 24 h, 4°C) and laminin (13 μg/ml, 24 h, 4°C) for 1 to 2 weeks in culture medium supplemented with retinoic acid (10 μM) and fetal calf serum (10%) but lacking neurotrophic factors and were regarded as precursors. Cells that have been grown in medium supplemented with nerve growth factor beta (NGF) (50 ng/ml, Tebu, Offenbach, Germany), cAMP (1 mM), 3-isobutyl-1-methylxanthine (0.5 mM), retinoic acid (10 μM), basic fibroblast growth factor (bFGF) (20 ng/ml), and fetal calf serum (15%) underwent differentiation toward neurons and were termed differentiated NT2 cells.

Cell incubation and Ca^{2+} measurements
Intracellular Ca^{2+} concentration changes were measured as recently described (Köller *et al*, 2001a, 2001c). Recombinant gp120 was added to the bath solution, which contained (mM) NaCl 150.0, KCl 4.0, $CaCl_2$ 2.8, $MgCl_2$ 1.0, HEPES 10.0, sucrose 10.0, pH adjusted to 7.4 by NaOH. Cells were incubated with gp120 in bath solution. In experiments using cell extract from HeLa-$T4^+$ cells expressing gp120 protein, the cell extract was dissolved in 2 ml bath solution, which was then used for incubation. Supernatants were used directly without dilution. After washing, cells were loaded with the cell-permeant form of the ratiometric dye Fura-2AM (5 μM) for 45 min. Pluronic F-127 (0.06%) was used to optimize dye uptake. After incubation and dye loading, the cells were perfused using the bath solution given above. Glutamate was added to the perfusion solution at a

final concentration of 500 μM. ATP was used at a final concentration of 1 mM. All Ca^{2+} measurements started 120 min after beginning of gp120 incubation.

Cells were alternately excited with light at 340- and 380-nm wavelengths and fluorescence were visualized using a CCD camera system (Merlin, Chromaphore, Duisburg, Germany). Because we were interested in intracellular Ca^{2+} changes upon glutamate application, we measured the fluorescence (F) after excitation with light at wavelengths of 340 and 380 nm and calculated the ratio ($F = F_{340}/F_{380}$), which reflects the intracellular Ca^{2+} concentration. For evaluation of gp120, glutamate, or ATP effects, the fluorescent change, dF, was calculated off line by subtracting the maximal fluorescence ratio (F_{max}) upon stimulation by the fluorescence ratio (F_0) prior to stimulation: $dF = F_{max} - F_0$.

Expression of virus proteins

HeLa-T4$^+$ cells (Maddon *et al*, 1986) were transfected with FuGENE^{TM} 6 (Roche Molecular Biochemicals) as described previously (Kammler *et al*, 2001) with expression vectors SVtat$^-$ rev$^-$ 3stop and SVcrev (1 μg each) coding for the HIV-1 protein gp120 and Rev. Plasmid sequences can be obtained on request (from H.S.). The medium was changed after 24 h and the cells were cultivated for another 24 h. The cells were scraped into the medium, sedimented at 1000 $\times$ g for 5 min, washed with phosphate-buffered saline (PBS), and resuspended in 100 μl PBS. Following three freeze- and-thaw cycles, cell extracts were kept frozen until their use in experiments. The effects of the respective supernatants and the cell extracts on the glutamate response of astrocytes were analyzed. Supernatants and extracts of cells that were transfected with one of the parental cloning vectors pSVT7 (Bird *et al*, 1987) served as control and did not affect the intracellular Ca^{2+} regulation.

Data analysis

All data are given as means and standard errors of the mean. Differences between cells preincubated with and without virus proteins were tested for significance using Student's t test.

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