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Functional expression of the seven-transmembrane HIV-1 co-receptor APJ in neural cells

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APJ is a recently described seven-transmembrane (7TM) receptor that is abundantly expressed in the central nervous system (CNS). This suggests an important role for APJ in neural development and/or function, but neither its cellular distribution nor its function have been defined. APJ can also serve as a co-receptor with CD4 for fusion and infection by some strains of human immunodeficiency virus (HIV-1) *in vitro*, suggesting a role in HIV neuropathogenesis if it were expressed on CD4-positive CNS cells. To address this, we examined APJ expression in cultured neurons, astrocytes, oligodendrocytes, microglia and monocyte-derived macrophages utilizing both immunocytochemical staining with a polyclonal anti-APJ antibody and RT–PCR. We also analyzed the ability of a recently identified APJ peptide ligand, apelin, to induce calcium elevations in cultured neural cells. APJ was expressed at a high level in neurons and oligodendrocytes, and at lower levels in astrocytes. In contrast, APJ was not expressed in either primary microglia or monocyte-derived macrophages. Several forms of the APJ peptide ligand induced calcium elevations in neurons. Thus, APJ is selectively expressed in certain CNS cell types and mediates intracellular signals in neurons, suggesting that APJ may normally play a role in signaling in the CNS. However, the absence of APJ expression in microglia and macrophages, the prinicpal CD4-positive cell types in the brain, indicates that APJ is unlikely to mediate HIV-1 infection in the CNS. *Journal of NeuroVirology* (2000) **6,** S61–S69.

Keywords: nervous system; signaling; chemokine receptor

Introduction

APJ is a seven-transmembrane (7TM) domain receptor that is abundantly expressed in human brain and spinal cord (O'Dowd *et al*, 1993; Matsumoto *et al*, 1996; Edinger *et al*, 1998). Its primary function is unknown and it has limited homology with other 7TM receptors ((O'Dowd *et al*, 1993; Matsumoto *et al*, 1996) and Table 1). Some 7TM receptors, notably CXCR4 and CCR5, are also expressed in the CNS and can serve as co-receptors with CD4 for fusion and entry by HIV-1, and APJ can also mediate fusion and entry by some HIV-1 strains in transfected cells *in vitro*. This suggests that APJ may also be a potentially important co-receptor for CNS HIV-1 infection (Edinger *et al*, 1998; Choe *et al*, 1998, Albright *et al*, 1999).

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The abundant expression of APJ RNA in the CNS raises the possibility that APJ may also normally mediate neuronal cell signaling. APJ signaling functions may play a role in nervous system development, since studies in fetal and adult rat brain indicate that APJ mRNA levels are high in fetal brain (O'Dowd *et al*, 1993). Matsumoto *et al* (1996) identified APJ RNA in adult human CNS in the corpus callosum, medulla, amygdala, hippocampus, substantia nigra, subthalamic nucleus, thalamus and spinal cord, with little expression in the striatum and cortex. RNA levels were highest in the corpus callosum and spinal cord, suggesting that APJ is primarily expressed in white matter tracts, possibly in glial cells. Furthermore, a natural peptide ligand for APJ, apelin, was recently isolated from bovine stomach extracts and demonstrated to induce signaling responses in cells transfected with APJ (Tatemoto *et al*, 1998). However, studies of APJ expression at the cellular level have been limited by

the lack of antibodies with which to detect APJ protein, and thus little is known about APJ expression and function in individual CNS cell types. In addition, no cell types from the CNS or from extra-neural sites that naturally express APJ have been examined for apelin-induced responses.

To better define the cellular distribution and function of APJ in the CNS, we generated a polyclonal antibody for APJ and used immunocytochemical labeling as well as RT–PCR to analyze APJ expression in CNS cells. Our results indicate that APJ is expressed predominantly in neurons and oligodendrocytes, at low levels in astrocytes, and not in microglia or monocyte-derived macrophages, the primary CD4-expressing cells in the brain. We detected calcium elevations in cultured human neurons exposed to apelin peptides, suggesting that APJ is functional in neurons. Thus, APJ may play an important role in neuronal signaling events in the brain, but likely does not serve as a co-receptor for HIV-1 infection in the CNS.

Results

APJ expression in neurons, oligodendrocytes and astrocyte, but not in macrophages or microglia

To determine which CNS cells expressed APJ, we examined purified cultures of human fetal astrocytes as well as microglia and oligodendrocytes from adult human brain for APJ RNA expression by RT–PCR, followed by Southern blotting. For analysis of human neurons, we utilized NT2.N neurons, a well-characterized model for developing human neurons with structural and functional features of primary CNS neurons (Andrews, 1984; Pleasure *et al*, 1992). We also tested primary human monocyte-derived macrophages as a model for perivascular brain macrophages, since they are phenotypically similar with respect to many cell surface markers and both are marrow-derived (Ulvestad *et al*, 1994). We consistently detected an RT–PCR signal for APJ in NT2.N neurons, astrocytes and oligodendrocytes (Figure 1). In contrast, no APJ band was detected in microglia or monocyte-derived macrophages, the only endogenous CD4-positive cells in the CNS (Figure 1). No band was

Table 1 Sequence homology between APJ and related genes

Gene grouping by cellular expression	*% overall homology with APJ*
Neuronal cell receptors	
Somatostatin receptor (type 3)	28
Somatostatin receptor (type 4)	28
Somatostatin receptor (type 1)	27
Opioid receptor (Mu type)	27
Opioid receptor (kappa type)	26
P2Y purinoceptor 9 (P2Y9)	28
P2Y purinoceptor 1 (ATP receptor)	27
P2Y purinoceptor 5	25
Mononuclear leukocyte chemotactic receptors	
CXCR-2 (high affinity IL-8-receptor)	30
CCR-1 (CC chemokine receptor)	27
CCR-3 (CC chemokine receptor)	28
CCR-4 (CC chemokine receptor)	29
CCR-10 (CC chemokine receptor)	28
Vascular endothelial receptors	
Angiotensin II receptor (type 1A) (AT1A)	31
Angiotensin II receptor (type 1B) (AT1B)	30
Bradykinin B2 receptor	26

Results of protein database homology search for APJ-related proteins (BLAST plus BEAUTY). All proteins with known cellular functions and $\geqslant 25\%$ amino acid homology are shown, and can be grouped into those associated with neuronal cell functions, lymphocyte chemotaxis, and vasoactive properties.

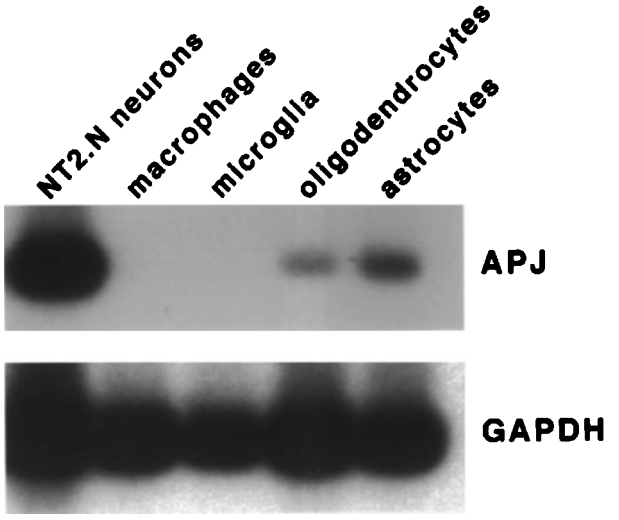


Figure 1 Detection of APJ RNA in neurons, oligodendrocytes and astrocytes. Total cellular RNA was prepared from NT2 neurons (NT2.N), oligodendrocytes, astrocytes, monocyte-derived macrophages and microglia and subjected to RT–PCR and Southern blotting to detect APJ, as described in Materials and methods. Fetal astrocyte cultures were >95% GFAP-positive and adult oligodendrocyte cultures were >95% galactosylceramide-positive, as judged by immunofluorescence labeling. The APJ PCR product size is 481 bp, confirmed by APJ plasmid control (not shown). As an internal control, amplification was carried out using primers for GAPDH.

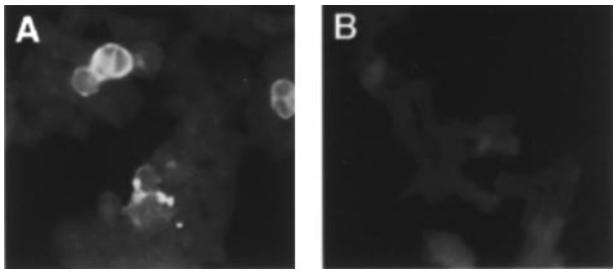


Figure 2 Immunofluorescence detection of APJ in transfected 293T human kidney cells. 293T cells were transfected with an APJ expression plasmid and stained 48 h later with polyclonal anti-APJ rabbit antiserum as described in Materials and methods. (**A**) APJ-transfected cells; (**B**) non-transfected cells. Magnification=400×.

seen when reverse transcriptase was omitted from the RT–PCR reaction (not shown), confirming that the signal detected represented RNA expression and not cellular DNA carryover.

To investigate APJ protein expression, we generated a polyclonal antibody, using the unique 29 amino acid N-terminal APJ peptide sequence as an immunogen. To confirm specificity, we used 293T cells transfected with an APJ expression plasmid (Edinger *et al*, 1998). As shown in Figure 2, non-transfected 293T cells did not stain with this antibody (Figure 2B). However, cells transfected with the APJ plasmid showed strong antibody labeling (Figure 2A), while the species-matched

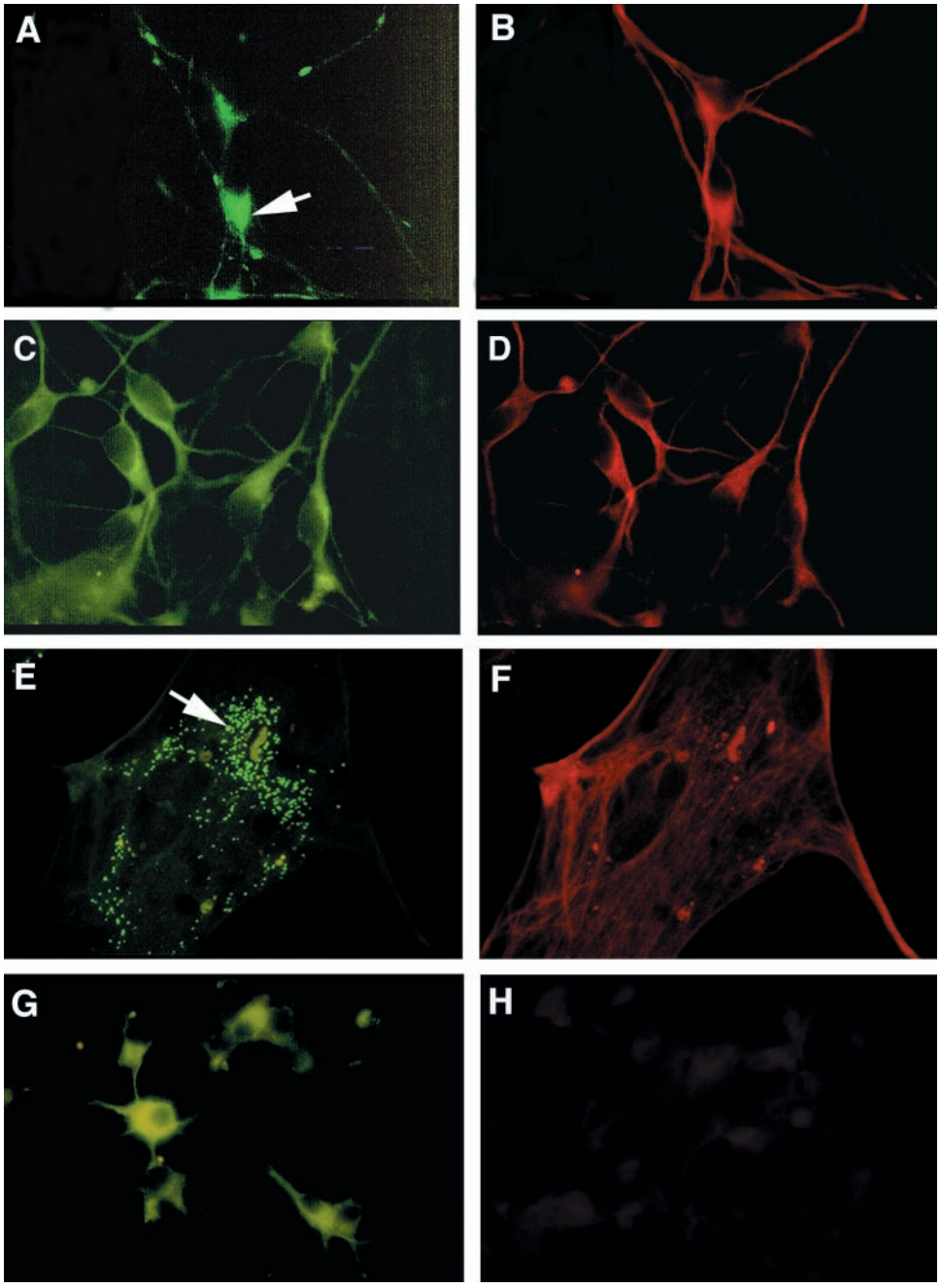
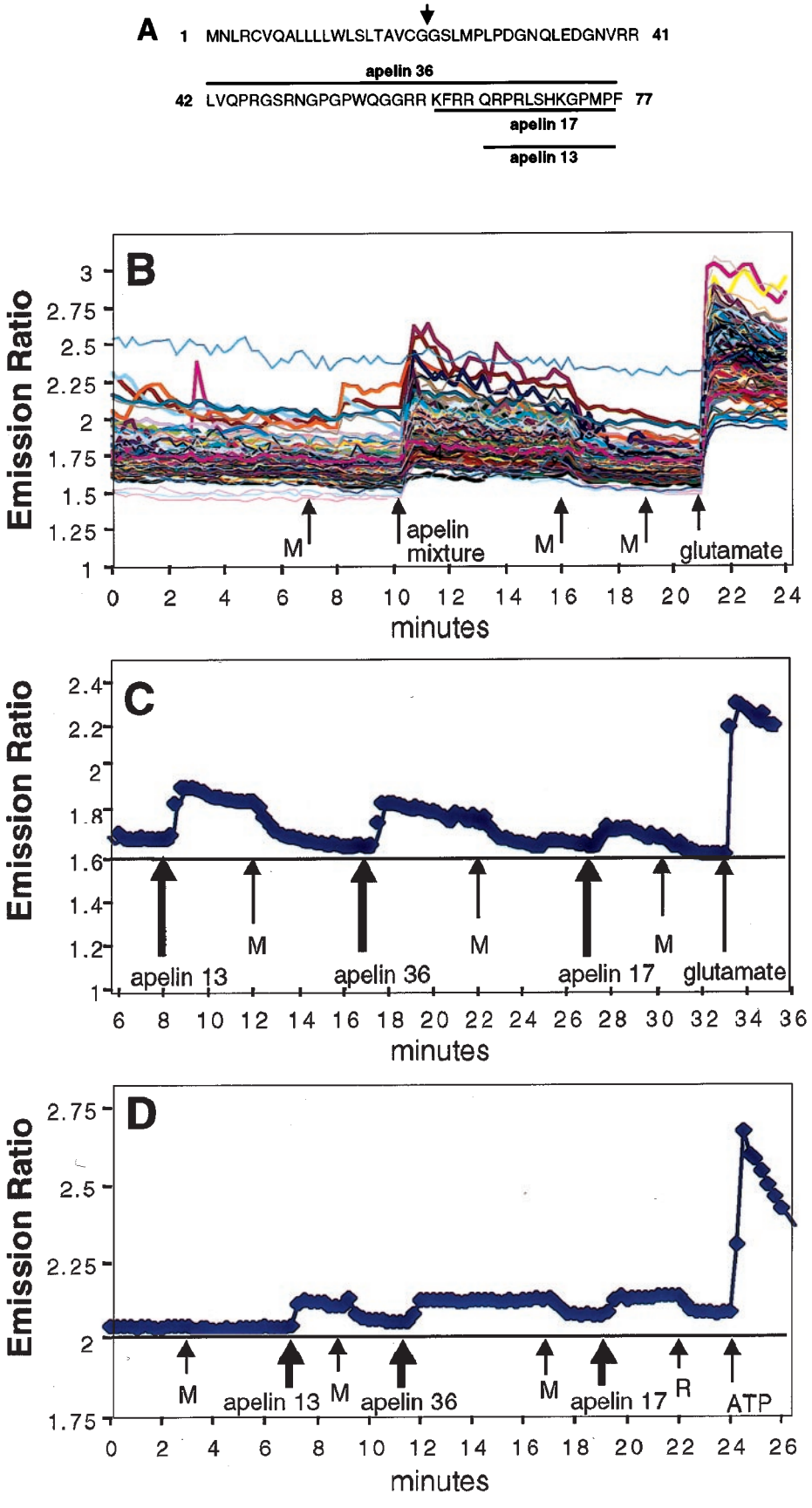


Figure 3 APJ staining of human neurons, oligodendrocytes and astrocytes. Neuronal cultures were co-stained with APJ antibody and microtubule-associated protein -2 (MAP-2), while astrocytes were similarly co-stained for APJ and glial fibrillary acidic protein (GFAP) as follows: (**A**) fetal neurons, APJ label; (**B**) fetal neurons, MAP-2 label; (**C**) NT2.N neurons, APJ label; (**D**) NT2.N neurons, MAP-2 label; APJ label; (**E**) fetal astrocytes, APJ label; (**F**) fetal astrocytes, GFAP label. Staining for APJ alone was performed in oligodendrocytes (**G**) and macrophages (**H**). Magnification=1000×.


A
1 MNLRCVQALLLLWLSLTAVCGGSLMPLPDGNQLEDGNVRR 41
apelin 36
42 LVQPRGSRNGPGPWQGGRR KFRR QRPRLSHKGPMPF 77
apelin 17
apelin 13
B
Emission Ratio
minutes
M
apelin mixture
M
M
glutamate
C
Emission Ratio
minutes
apelin 13
M
apelin 36
M
apelin 17
M
glutamate
D
Emission Ratio
minutes
M
apelin 13
M
apelin 36
M
apelin 17
R
ATP

control antibody remained negative (not shown). APJ antibody labeling was completely blocked by pre-incubation of the antibody with the APJ peptide, and only transfected 293T cells demonstrated APJ RNA expression by RT–PCR (not shown). Taken together, these results demonstrated the specificity of the APJ antibody for detection of APJ in fixed cells.

To address APJ protein expression in neurons and glial cells, we then examined cultures of primary human fetal neurons, NT2.N neurons, astrocytes, oligodendrocytes, microglia and monocyte-derived macrophages with the APJ antibody. As shown in Figure 3, both human fetal neurons (Figure 3A,B) and human NT2.N neurons (Figure 3C,D) stained with the APJ antibody. In both human fetal and NT2.N neuronal cultures, greater than 95% of cells were positive for APJ. Furthermore, APJ showed a consistent pattern of distribution in neurons that was most prominent in the cytoplasm near the axon hillock (Figure 3A, arrow). In contrast to neurons, less than 5% of fetal astrocytes were positive for APJ (Figure 3E,F). In the few astrocytes where APJ was detected, a punctate intracellular staining pattern was seen (Figure 3E, arrow).

Oligodendrocytes were also greater than 95% positive for APJ, although unlike neurons, staining was diffuse throughout the cytoplasm (Figure 3G). APJ staining in all cells was completely blocked by the APJ peptide, and in all cases no signal was seen with the species matched control antibody (not shown).

When we stained primary human microglia (not shown) and monocyte-derived macrophages (Figure 3H), we found no APJ expression in either of these CD4-positive cell types. Thus, APJ immunoreactivity in each neural cell type was completely concordant with our detection by RT–PCR and indicates abundant APJ in fetal neurons and adult oligodendrocytes, limited expression in astrocytes, and no expression in macrophages or microglia.

Intracellular calcium elevations induced by the APJ ligand apelin in neurons

Because we detected differential APJ expression in specific CNS cell types, we next wished to determine whether APJ was functional in neural cells. We tested whether changes in intracellular calcium levels could be detected in NT2.N neurons after stimulation by apelin, recently identified as a ligand for APJ (Tatemoto *et al*, 1998). We utilized three different apelin peptides (Figure 4A), that have been demonstrated to induce functional responses including pH changes in APJ-transfected cells (Tatemoto *et al*, 1998). Apelin-36 (the carboxy-terminal 36 amino acids), and apelin-17 (the carboxy-terminal 17 amino-acids), and apelin- 13 (the carboxy-terminal 13 amino-acids) have been shown to possess increasing biological activity associated with decreasing size (Tatemoto *et al*, 1998). Apelin-induced signaling has not been addressed, however, in cells naturally expressing APJ.

Exposure of NT2.N neurons to a mixture of all three peptides produced a sharp rise in intracellular calcium concentrations (Figure 4B; apelin mixture). We then tested each peptide individually and found that each form of apelin was active in NT2.N neurons (Figure 4C,D). No response was seen to mock injection (M, vehicle only) or to the β-chemokine RANTES (R, Figure 4D), while a brisk response was seen to glutamate (Figure 4C) and ATP (Figure 4D). While we did not observe consistent differences in the magnitude of the calcium response to the three forms of apelin, we did find, with repeated exposure of cells to peptide, that the responses were sequentially lower with each successive application. This occurred regardless of the order of admininstration, suggesting partial desensitization of the response. Thus, neurons demonstrate calcium responses to apelin, indicating that APJ is functional with respect to its ability to induce a signal in these cells in response to its ligand. In contrast, no calcium elevations were seen in astrocytes treated similarly with apelin peptides (data not shown).

Discussion

In this study we showed that APJ is abundantly expressed in fetal neurons and mediates changes in intracellular calcium levels in response to its

Figure 4 APJ ligand-induced calcium flux in NT2.N neurons. NT-2N neurons were loaded with 2.5 μM fura-2/AM and prepared as described in the Materials and methods. (**A**) Preproapelin and the apelin peptide sequences used in signaling experiments. The sequence was derived from Tatemoto *et al* (1998). Arrow indicates the site of predicted cleavage of a putative signal peptide. (**B**) Simultaneous injection of NT-2N cells with three forms of the APJ ligand: apelin 36 (60 nM), apelin 17 (120 nM), and apelin 13 (170 nM) resulted in a change in the intracellular [Ca^{2+}], expressed as single cell tracings of the emission ratio at 510 nM, following excitation at 340 and 380 nM. Mock injection (M) with vehicle alone induced no change in intracellular [Ca^{2+}], and glutamate (0.7 mM) and ATP (666 nM) served as positive controls. These data are representative of three replicate experiments. (**C, D**) NT2-N neurons were exposed separately to increasing doses each of the three forms of apelin, each of which induced changes in intracellular calcium levels. Doses are as follows: (**C**) apelin 13 (170 nM), apelin 17 (120 nM) and apelin 36 (60 nM); (**D**) apelin 13 (43 nM), apelin 17 (30 nM) and apelin 36 (15 nM). (**D**) RANTES (R, 60 nM) and ATP (666 nM) served as negative and positive control, respectively. Data in **C** and **D** are expressed as the average of 20–30 single-cell measurements.

recently identified ligand, apelin. Based upon immunofluorescence detection of APJ protein, glial cell expression is mainly in oligodendrocytes, with limited expression in astrocytes. In contrast, APJ is not expressed in macrophages or microglia, the only CD4-positive cells and the major HIV-1 reservoir in the CNS. These results indicate that APJ may serve important neuronal and glial signaling functions, and suggest that it does not serve as a co-receptor for HIV-1 infection in the CNS.

Previous studies of APJ function utilized transfected cells, and our study is the first demonstration of APJ function in naturally-expressing cells. Our demonstration of high expression in neurons, along with previous studies showing RNA expression in the brain suggest a major role for APJ in neuronal signaling in the CNS. In fetal neurons APJ protein was localized predominantly to the area of the axon hillock, similar to what has been described for the 7TM chemokine receptor, CCR3, in fetal macaque neurons (Klein *et al*, 1999). We also found APJ expression in dendritic projections, although more weakly. This suggests that APJ may mediate specific axonal functions and that neurons may receive APJ-mediated signals from other cells along their neuritic projections.

Glial cell expression of APJ was predominately in oligodendrocytes, and expression was diffuse throughout the cell. The abundant expression of APJ in oligodendrocytes as well as in neuronal axons, which are the major cellular components of white matter, may account for the predominance of APJ in certain white matter tracts within the brain and spinal cord (Matsumoto *et al*, 1996; Edinger *et al*, 1998). We recently found relatively high levels of APJ mRNA in adult human corpus callosum, spinal cord and medulla, which contain major white matter tracts, and lower-level expression in hippocampus, substantia nigra, subthalamic nucleus, thalamus, cerebellum and cortex which are major gray matter areas (Edinger *et al*, 1998). The relatively lower expression of APJ RNA in gray matter areas, which contain neuronal cell bodies and dendritic projections, suggests that APJ may be expressed in only certain neuronal subsets *in vivo* or that neuronal APJ expression may vary between fetal and adult tissue. Finally, unlike neurons and oligodendrocytes, the expression of APJ in fetal astrocytes is infrequent (5% of cells) and intracellular, suggesting that in astrocytes in developing CNS, it is unlikely to serve as a receptor for extracellular ligand. Consistent with this, we have been unable to demonstrate signaling in fetal astrocytes by apelin.

Notably, we found no APJ expression in adult microglia or monocyte-derived macrophages, the only endogenous CD4-expressing cells within the CNS. Since cells of the macrophage/microglia lineage are the only sites of productive HIV-1 infection within the CNS, and since all HIV-1 isolates that can utilize APJ as an infection cofactor require CD4 (Edinger *et al*, 1998; Choe *et al*, 1998), we believe, therefore that APJ has no role in macrophage/microglia infection in the CNS. Whether HIV envelope binding to neuronal or glial APJ occurs in the absence of CD4 with subsequent functional consequences or with establishment of glial (astrocytic) infection is unknown, however.

The normal biological function of APJ in the CNS is currently unknown, although its sequence predicts a 7TM topology resembling G-protein coupled 7TM receptors (GPCRs) (see Table 1). APJ was first cloned by O'Dowd *et al* (1993) from human genomic DNA, and further analysis revealed that APJ is expressed across many species, including chimpanzee, monkey (*Cercopithecus aethiops*) and rat, suggesting an essential conserved function *in vivo*. Although our database search for human genes encoding proteins with APJ sequence similarity did not produce a candidate protein with significant ($>30\%$) overall homology to APJ, several interesting features of the retrieved protein sequences were apparent. First, most of the proteins (19 of 20), including APJ, express a G-protein coupled receptor signature. And second, most of the proteins broadly fall into three categories: proteins associated with neuronal cell function, with leukocyte chemotaxis, and with blood vessel constriction or dilatation (Table 1).

Other G-protein coupled receptors in the nervous system modulate specific neuronal cell functions, including neurotransmitter metabolism and axonal growth (O'Dowd *et al*, 1991; Gilman, 1995; Vancura *et al*, 1998). For example, CXCR4, which like APJ functions also as a cofactor for HIV-1 entry into cells, was recently demonstrated on human neurons, macrophages, microglia (Lavi *et al*, 1997, 1998), and astrocytes (Tanabe *et al*, 1997). Studies of CXCR4-knockout mice demonstrate that it plays an important role in neuronal pathfinding as well as blood vessel formation (Ma *et al*, 1998; Zou *et al*, 1998; Tachibana *et al*, 1998). Based on these CXCR4 studies and our demonstration of APJ ligand-induced calcium elevations in neurons, we speculate that APJ may also play a role in neuronal signaling in the developing nervous system, and possibly in neuronal pathfinding. Consistent with this, in preliminary studies we have detected apelin RNA in oligodendrocytes, but not neurons, macrophages, microglia or astrocytes, suggesting cell-specific expression of apelin in the CNS (data not shown). However, defining the role for APJ will depend on determining its distribution throughout the CNS during development, determining the expression patterns of apelin in the CNS, and ultimately determining the effects of loss of APJ expression in the CNS.

Materials and methods

Cells and cultures

NT2.N neurons NT2.N neurons were generated from NTera 2/cl.D1 cells as previously described (Pleasure *et al*, 1992). Briefly, 2.7×10^6 cells were seeded in a 75 cm^2 flask and exposed to 10 μM retinoic acid for 5 weeks, followed by replating at low density (1 : 6) for 7 days. Cells were then finally replated onto glass coverslips coated with Matrigel (Collaborative Biomedical Products, Bedford, MA, USA) in DMEM with 5% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml), 1 μM cytosine arabinoside, 10 μM fluorodeoxyuridine and 10 μM uridine (Sigma) at a density of 2×10^5 cells per cm^2. Cultures were $>99\%$ neurons as judged by microtubule-associated protein-2 (MAP-2) staining (Pleasure *et al*, 1992).

Human fetal astrocytes Human fetal astrocytes, provided by B Wigdahl (Pennsylvania State University, USA), were obtained from 10–17 week old fetuses in accordance with NIH guidelines. After dissection, brain tissue was mechanically dissociated, incubated in 0.5% Trypsin/DNase (2 ug/ml) and cultured in DMEM containing 10% fetal bovine serum, L-glutamine (2 mM), penicillin (100 U/ml) and streptomycin (100 U/ml). After several days, flasks were gently shaken for 48 h, and the remaining adherent cells were cultured in astrocyte selection medium (DMEM without glucose, 10% FBS, 25 mM D-sorbital and 1 mM L-leucine methyl ester) for 2 weeks prior to use. Cultures were stained for glial fibrillary acidic protein (GFAP) to assess culture purity, which was typically $>95\%$ astrocytes.

Human fetal neurons Mixed human fetal neuronal/glial cells were provided by A Nath (University of Kentucky, USA) and were cultured from human brain tissue from 12–15 week-old fetuses in accordance with NIH guidelines. After dissection, tissue was mechanically disrupted by aspiration through a 19-gauge needle, rinsed in Eagle's minimal essential medium (MEM) and cultured in MEM containing 10% fetal bovine serum (FBS), L-glutamine (2 mM) and gentamicin (5 μg/ml).

Human macrophages Monocyte-derived macrophages (MDM) were isolated from peripheral blood mononuclear cells of healthy volunteers by selective adherence as previously described (Collman *et al*, 1989). Cells were cultured on poly-L-lysine coated glass coverslips for 7 days prior to immunofluorescence staining.

Human oligodendrocytes and microglia Brain-derived microglia and oligodendrocytes were isolated from fresh adult human brain tissue obtained from temporal lobe resections from patients with medication-resistant epilepsy as previously described (Strizki *et al*, 1996; Albright *et al*, 1996). Tissue was mechanically dissociated, treated with trypsin, and subjected to differential centrifugation to separate glial cell types, and purity of the microglial cultures was $>95\%$, as judged by uptake of Di-I-Acylated LDL (Goldstein *et al*, 1979). Oligodendrocyte cultures were $>99\%$ pure, based on immunofluorescence labeling with anti-galactosylceramide antibody (Albright *et al*, 1996).

Procedures and experimental protocols involving the use of human tissues were approved by the Institutional Review Committee of the University of Pennsylvania in compliance with NIH guidelines.

RT–PCR and Southern blotting

Total RNA was prepared from 1×10^6 cells (RNeasy kit; Qiagen, Inc., Chatsworth, CA, USA), treated with RNase-free DNase I (40 U/10 μg of RNA; Boehringer Mannheim, Indianapolis, IN, USA) for 30 min at room temperature in the presence of 200 U/ml RNasin (RNase inhibitor; Boehringer Mannheim). cDNA was synthesized from 0.5 μg of total RNA with random hexamers and SuperScript II RNase-reverse transriptase (GIBCO BRL, Grand Island, NY, USA) followed by heat inactivation and RNase H treatment at 37°C for 20 min. APJ amplification utilized sense (5′-TACACAGACTGGAAATCCTCG-3′) and antisense (5′-TGCACCTTAGTGGTGTTCTCC-3′) primers that yield a 481 bp product. APJ PCR conditions were: 33 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, and extension at 72°C for 1 min 30 s. Products were resolved on 1.5% agarose gels and subjected to Southern blot with a ^{32}P-labeled internal probe (5′-ATGTTGACGAAGATGAGGTAGC-3′). As a control, GAPDH amplification was performed in parallel as previously described (Choe *et al*, 1997).

APJ polyclonal antibody

To generate an APJ-specific antibody, we utilized an amino-terminal APJ peptide (1-MEEGGDFDNYYGADNQSECEYTDWKSSGA-29) that showed no homology with other peptide sequences (BLASTP plus BEAUTY search with BCM Search Launcher). APJ-reactive polyclonal serum was generated by monthly injections of KLH-conjugated peptide (100 μg/injection) into New Zealand White rabbits, followed by pooled blood collection at 2, 4 and 6 months. Serum titers were determined by ELISA against the unconjugated peptide, and high titer samples were pooled and purified by affinity chromatography utilizing the unconugated APJ peptide. Final titers were $\geqslant 82\,600$. The polyclonal antibody was determined to have no cross-reactivity against CCR1, CCR5, CCR8 or CXCR4 by FACS analysis in transfected 293T cells (data not shown).

Immunofluorescence labeling
Cells were grown on poly-L-lysine-coated glass coverslips and fixed with ice-cold ethanol/acetic acid (95 : 5) or 4% paraformaldehyde in PBS for 20 min. They were sequentially blocked (10% goat serum/PBS for 30 min at room temperature), incubated with rabbit polyclonal anti-APJ antibody (3 μg/ml for 60 min at room temperature), washed and incubated with biotinylated swine anti-rabbit immunoglobulin [5 μg/ml (DAKO; Carpintera, CA, USA)] for 60 min at room temperature, followed by FITC-conjugated streptavidin (13.3 μg/ml in 10% goat serum, 25% FBS). In some experiments, Evan's Blue (0.01%; Sigma; St. Louis, MO, USA) was included as a red counterstain. As a negative control, rabbit polyclonal serum against an irrelevant antigen (Incstar, Stillwater, MN, USA) was used at the same concentration. For double-labeling of astrocytes, mouse anti-GFAP monoclonal antibody (clone G-A-5, 1 : 400 dilution; Sigma) was included with the APJ antibody. Detection of the primary APJ antibody was with FITC and GFAP antibody was detected with TRITC (tetramethylrhodamine isothiocyanate)-conjugated anti-mouse antibody (DAKO).

Apelin-induced calcium elevations in neurons
Intracellular calcium measurements $[Ca^{2+}]_i$ were performed as previously described (Albright *et al*, 1999). Briefly, 3-day-old NT2.N neurons cultured at a density of 1×10^5 cells/cm^2 on coverslips were mounted in a perfusion chamber (RC-21B; Warner Instrument Corp., Hamden, CT, USA) that was mounted on an upright epifluorescence microscope (Optiphot; Nikon, Tokyo, Japan). Cells were loaded with 2.5 μM fura-2/AM in 0.02% Pluronic F-127 for 30 min in standard recording medium (Itoh *et al*, 1998) and exposed to apelin peptides in DMEM with 10% FBS, while emission fluorescent images at 510 nm were recorded during excitation with 340 and 380 nm wavelength light. Analysis of data was performed as previously described (Albright *et al*, 1999).

Apelin peptides were produced by solid-phase chemical synthesis based on the tBoc αNH2-protection strategy and purified by reversed-phase HLPC (Clark-Lewis *et al*, 1997).

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