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HIV-1 Tat causes cognitive deficits and selective loss of parvalbumin, somatostatin, and neuronal nitric oxide synthase expressing hippocampal CA1 interneuron subpopulations

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Abstract

Memory deficits are characteristic of HIV-associated neurocognitive disorders (HAND) and co-occur with hippocampal pathology. The HIV-1 transactivator of transcription (Tat), a regulatory protein, plays a significant role in these events, but the cellular mechanisms involved are poorly understood. Within the hippocampus, diverse populations of interneurons form complex networks; even subtle disruptions can drastically alter synaptic output, resulting in behavioral dysfunction. We hypothesized that HIV-1 Tat would impair cognitive behavior and injure specific hippocampal interneuron subtypes. Male transgenic mice that inducibly expressed HIV-1 Tat (or non-expressing controls) were assessed for cognitive behavior or had hippocampal CA1 subregions evaluated via interneuron subpopulation markers. Tat exposure decreased spatial memory in a Barnes maze and mnemonic performance in a novel object recognition test. Tat reduced the percentage of neurons expressing neuronal nitric oxide synthase (nNOS) without neuropeptide Y immunoreactivity in the stratum pyramidale and the stratum radiatum, parvalbumin in the stratum pyramidale, and somatostatin in the stratum oriens, which are consistent with reductions in interneuron-specific interneuron type 3 (IS3), bistratified, and oriens-lacunosum-moleculare interneurons, respectively. The findings reveal that an interconnected ensemble of CA1 nNOS-expressing interneurons, the IS3 cells, as well as subpopulations of parvalbumin- and somatostatin-expressing interneurons are

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preferentially vulnerable to HIV-1 Tat. Importantly, the susceptible interneurons form a microcircuit thought to be involved in feedback inhibition of CA1 pyramidal cells and gating of CA1 pyramidal cell inputs. The identification of vulnerable CA1 hippocampal interneurons may provide novel insight into the basic mechanisms underlying key functional and neurobehavioral deficits associated with HAND.

Keywords

NeuroAIDS; Neurodegeneration; Spatial memory; Interneuron specific interneuron type 3 (IS3); Oriens-lacunosum-moleculare cell (O-LM); Bistratified cell

Introduction

Following the discovery of human immunodeficiency virus type 1 (HIV-1) and acquired immunodeficiency syndrome (AIDS), a profile of progressive neurological symptoms came to be associated with HIV-1 infection (Snider et al. 1983; Levy et al. 1985), now collectively referred to as HIV-associated neurocognitive disorders (HAND; Antinori et al. 2007; McArthur et al. 2010). Although there is variation among patients, common symptoms include deficits in attention, learning, memory, and impaired motor function. The neurocognitive aspects of HAND can be severe enough to interfere with independent living (Antinori et al. 2007). Notably, combined antiretroviral therapy (cART) does not fully ameliorate these symptoms (Sacktor et al. 2002; Tozzi et al. 2005; McArthur 2004; Ellis et al. 2007).

HIV may contribute to the progressive neurocognitive impairment in combination antiretroviral therapy (cART)-controlled patients through the continued production of neurotoxic HIV-1 proteins from cellular reservoirs within the central nervous system (CNS) (Johnson et al. 2013). Shortly after infection, HIV-1 can enter the brain and establish central reservoirs (Lane et al. 1996; An et al. 1999), infecting microglia or astrocytes, but not neurons (He et al. 1997; Brack-Werner 1999; Kramer-Hämmerle et al. 2005; Li et al. 2011; Churchill et al. 2009). Uninfected cells can be damaged by viral proteins, as well as by the host neuroinflammatory response to virions and free viral proteins (Nath et al. 1996; Kruman et al. 1998; Nath et al. 1999; Aksenov et al. 2001; Soulas et al. 2009; Zhou and Saksena 2013; Nath and Steiner 2014). Among these neurotoxic factors, the HIV-1 transactivator of transcription (Tat) protein is critical for HIV replication (Zhou and Saksena 2013; Nath and Steiner 2014) and can be secreted by infected cells (Ensoli et al. 1990; Thomas et al. 1994; Rayne et al. 2010; Debaiseux et al. 2012) even in the presence of cART (Johnson et al. 2013). Furthermore, Tat can bind to a large number of extracellular receptors due to its highly basic domain (Philippon et al. 1994; Zhou and Saksena 2013), penetrate and traverse the plasma membrane, and interact with a number of intracellular factors (Debaiseux et al. 2012).

Despite regional differences in CNS vulnerability to HIV-1 (Nath 2015), few studies have examined why some brain regions and neuronal types are preferentially susceptible to the virus. A number of behavioral deficits, including attenuation of spatial learning and memory, which are common features of HAND, can be recapitulated by Tat exposure and may be

attributed to cellular and functional deficits in the hippocampus (Li et al. 2004; Fitting et al. 2006; 2013). Long-term potentiation (LTP) is lost when Tat is injected intrahippocampally or expressed endogenously (Li et al. 2004; Behnisch et al. 2004; Fitting et al. 2013). Despite the pronounced loss of LTP and deficits in behavior, CA1 pyramidal cells showed only a modest decrease in the density of apical dendritic spines, but no loss in dendrite length or synaptic integrity at the ultrastructural level, and no alterations in the level of proteins involved in excitatory glutamatergic pre- and post-synaptic function (Li et al. 2004; Fitting et al. 2013). By contrast, there were selective alterations in proteins involved in inhibitory γ-aminobutyric acid-ergic (GABAergic) synaptic function, including a significant decline in synaptotagmin 2 (Syt2) expression (only within stratum radiatum) and marked increases in the expression of gephyrin throughout CA1 (Fitting et al. 2013). These findings concur with human gene array data which show a high degree of correlation between the expression of genes associated with inhibitory GABAergic synaptic transmission, including *GAD1* and *GABAR1*, and the severity of HAND, but showed less robust correlations with genes associated with glutamatergic neurotransmission (Gelman et al. 2012).

GABAergic interneurons are a diverse population of specialized cells responsible for much of the processing that occurs in the hippocampus. With estimates of at least 21 distinct interneuron subtypes in CA1, there are numerous potential targets for the development of hippocampal pathology (McBain and Fisahn 2001; Klausberger and Somogyi 2008; Lovett-Barron and Losonczy 2014). While numerous studies have examined the effects of HIV-1 on glutamatergic transmission in the hippocampus, few studies have assessed whether GABAergic synapses are altered. Pathology within specific GABAergic interneuron subpopulations, and the hippocampal interneuron network as a whole, has been demonstrated in a number of neurological disorders (Korotkova et al. 2010; Tóth et al. 2010; Hazra et al. 2013; Levenga et al. 2013), some of which produce behavioral deficits similar to those observed in experimental models of neuro-acquired immunodeficiency syndrome (neuroAIDS) (Carey et al. 2012; Fitting et al. 2013). In HIV models, reductions in the number of hippocampal neurons expressing parvalbumin (PV) in CA3 and somatostatin (SST) in CA1 have been observed (Masliah et al. 1992; Fox et al. 1997); however, since multiple neuron types in CA1 and CA3 express these markers, the identities of the affected interneuron subtypes remain unclear.

Based on the collective findings above, we hypothesized that specific interneuron subpopulation(s) might be preferentially vulnerable to HIV-1 Tat and that deficits in one or more interneuron subtypes following Tat exposure would coincide with impaired cognitive performance on hippocampus-dependent behavioral tasks. Our data show that HIV-1 Tat exposure was accompanied by memory impairment in mice and resulted in a selective decrease in the proportion of nNOS positive (nNOS+), neuropeptide Y negative (NPY−) neurons in both the stratum pyramidale and stratum radiatum, as well as decreases in somatostatin-positive (SST+) neurons in the stratum oriens and parvalbumin positive (PV+) neurons in the stratum pyramidale.

Materials and methods

Animals and treatments

Male mice were used exclusively throughout these studies. Inducible, glial fibrillary acidic protein-driven transgenic Tat mice (aged 60–80 days) were used to identify the effects of HIV-1 $Tat_{1–86}$ on hippocampal interneuron subpopulations and associated behavior (Bruce-Keller et al. 2008). In order to induce Tat expression, mice expressing the *tat* and *rtTA* transgenes (Tat+) were fed doxycycline-containing chow (DOX; 6 mg/g, Harlan, Indianapolis, IN) for 12–14 days depending on the experiment. Control mice lacking the *tat* transgene, but expressing the *rtTA* gene (Tat−), were given the same DOX treatment. Animal procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee and are in accordance with Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

Barnes maze

Tat− (n = 13) and Tat+ (n = 8) mice were assessed for spatial learning and motor function in a Barnes maze test (Barnes 1979). Mice consumed DOX chow for 7–8 days prior to 1 day of prehabituation, followed by 4 days of testing and 1 day of probe trials. For prehabituation, mice were placed in the escape hole (positioned in a randomly assigned location) for 2 min, then were placed in the brightly lit center of a Barnes maze (91 cm diameter, 90 cm height, with 20 holes each 5 cm diameter; Stoelting Co., Wood Dale, IL), and guided to the assigned escape hole where they remained for 2 min, then were placed under a glass cylinder next to the escape hole, and allowed up to 3 min to volitionally enter (mice that did not enter the hole were guided in and remained for 2 min). On testing days (four trials per day over 4 days), mice were placed in the brightly lit center of the Barnes maze and were allowed up to 3 min to enter the escape hole. Mice that did not enter the hole were gently guided to the hole and allowed to remain for 2 min. Shorter latencies to find the escape hole, a greater proportion of time spent in the correct quadrant of the maze, and fewer errors were considered indices of greater learning (Camara et al. 2013). On day 5, a reversal probe trial was conducted wherein the maze was turned 180° such that the correct goal box was on the opposite end of the Barnes maze table, and the proportion of time spent in the new target quadrant, latency to find the new escape hole, and number of errors made were assessed. Distances and velocities traveled were used as an index of motor behavior.

Novel object recognition

Tat− (n = 14) and Tat+ (n = 14) mice were assessed for neurocognitive function in a novel object recognition test (Ennaceur and Delacour 1988; Dere et al. 2007) after 14 days on DOX. Briefly, mice were habituated to a testing room, placed in an open field (35 × 40× 40 cm; Stoelting Co., Wood Dale, IL), and allowed to explore two identical round objects (plastic toys in the shape of kiwis, oranges, potatoes, or tomatoes; each ~6 × 3 cm) for a 10-min training trial. After a 4-h inter-trial interval, one of the previously explored objects was replaced with a novel cone-shaped object of similar size (a plastic toy in the shape of a half ear of corn or a half-pickle), which mice were allowed to explore for a 10-min retention trial. Across subjects, replacement of the familiar object on the left vs. right side of the apparatus was counterbalanced to control for potential side-preferences. For each trial, the time spent

investigating the object in the novel position, as a function of the entire time spent investigating, was calculated: [(novel object time − familiar object time)/(novel object time + familiar object time)]. To assess motor behavior, the overall distance traveled, as well as the time and frequency spent rearing were recorded.

Vision testing

Tat− (n =6) and Tat+ (n=6) mice were assessed for visual function based on prior methods (Wersinger et al. 2002) prior to DOX exposure, and at 7 and 14 days following DOX exposure. In brief, mice were suspended approximately 12 in. above a vertical ring-stand and were gently lowered with the ring-stand approximately 2 in. from the left or right visual field (close enough to allow visual, but not whisker, contact with the ring-stand). The left and right visual fields were assessed consecutively for each mouse. Visual responding was considered positive when mice reached with the forepaws for the rod when presented to both the left and right side (a response to only one visual field would have been considered a negative response). Two observers rated visual responses.

Immunohistochemistry

Brains were fixed in 4 % paraformaldehyde using transcardial perfusion, removed, halved, and embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA). Hemisected brains were sectioned in the sagittal plain at a thickness of 16 μm for analysis of interneuron populations or in the coronal plane at a thickness of 40 μm to determine the reference volume using stereology. A Leica CM1850 cryostat was used to cut the sections (Leica Biosystems, Buffalo Grove, IL). Sections were mounted on slides and then stored at −80 °C until use. Tissue sections for interneuron analyses were blocked, then incubated with individual primary antibodies (anti-PV, Synaptic Systems, Cat. No. 195 004, guinea pig polyclonal, 1:1200; anti-nNOS, Abcam, ab1376, goat polyclonal, 1:5000; anti-NPY, Abcam, ab30914, rabbit polyclonal, 1:100; anti-SST, Santa Cruz Biotechnology, sc-13099, rabbit polyclonal, 1:50; anti-NeuN, Chemicon, MAB377, mouse monoclonal, 1:500) for 18 h at 4 °C, and rinsed in phosphate-buffered saline (PBS). Solutions containing appropriate, fluorescently labeled secondary antibodies (goat anti-guinea pig IgG-Cy3, Abcam ab6965-100, 1:500; Abcam ab102370, 1:500; donkey anti-goat IgG-Alexa Fluor® 488, Invitrogen, A11055, 1:500; goat anti-rabbit IgG-Alexa Fluor® 647, Invitrogen A21244, 1:500; donkey anti-mouse IgG-Alexa Fluor® 594, Invitrogen, A21203, 1:500; goat anti-mouse IgG-488 Oregon green, Molecular Probes, 0-6380, 1:500) were placed onto tissue sections for 2 h at room temperature. Slices were then rinsed 3 × 10 min in PBS. All tissue sections were incubated in PBS/Hoechst solution (0.5 μg/ml, Invitrogen, H3570) for 5–10 min, repeatedly rinsed in PBS, and mounted in ProLong Gold Antifade reagent (Invitrogen, P36930).

Reference volume analysis

Hoechst-stained 40-μm serial sections were sampled through the entire hippocampus. From a random starting position, sections at evenly spaced intervals were sampled to obtain an unbiased stereological assessment of hippocampal volume (Gundersen et al. 1988). In short, sections from halved brains were taken from Tat− (n = 6) and Tat+ (n = 5) mice and imaged using a Zeiss Axio-Observer Z1 microscope, MRm digital camera, and a 10× objective

(Zeiss, Oberkochen, Germany). Montages of the entire left hemisphere of the brain (5 × 6) were obtained utilizing a motorized stage encoder (Zeiss) and a computerized tile-reconstruction method (Zeiss, Axio-Vision software). Hippocampal volume was then estimated by overlaying a standardized grid over the image of the brain and performing a point count analysis (Gundersen et al 1988; Mouton 2002).

Imaging

Hippocampi were imaged using a Zeiss LSM 700 microscope at 20× magnification (Zeiss, Oberkochen, Germany). Neurons, as noted by the presence of NeuN immunoreactivity within the nucleus, were counted in stratum oriens, stratum pyramidale, stratum radiatum, and stratum lacunosum-moleculare of CA1. Immunoreactivity for each protein was noted independently, and the markings were overlaid to confirm co-expression of proteins without bias. From 4 to 10 sections (typically 7 to 8) were analyzed and averaged per animal, and 6 mice per experimental group were used for all experiments. All cell count data are presented as a percent of the total NeuN+ population in each layer. To examine the potential effects of the Tat transgene on the neuritic processes, densitometric analysis of PV+ dendrites and SST + axons was performed using ImageJ (NIH, Bethesda, MD). In addition, dendritic varicosities of PV+ interneurons were quantified on the five most pronounced dendrite segments from each image within stratum radiatum from 2 to 3 sections per animal. Dendrites not reaching at least 50 μm in length or discontinuous dendrites were not analyzed.

Statistical analyses

Behavioral endpoints were recorded and digitally encoded by an ANY-maze animal tracking system (Stoelting Co., Wood Dale, IL) and assessed via repeated-measures analyses of variance (ANOVA) with novel object recognition trial (training trial or retention trial) or Barnes maze testing day (days 1–5) and mouse genotype (Tat− or Tat+) as the respective within-, and between-, subjects factors. Tukey's honestly significant difference post hoc tests were used to assess group differences following main effects. Interactions were delineated via simple main effects and main effect contrasts with alpha corrected for multiple comparisons. Immunohistochemical endpoints were assessed with one-tailed Student's *t* tests. Effects were considered significant when $p<0.05$.

Results

HIV-1 Tat expression impairs mnemonic performance

Inducing HIV-1 Tat expression in the CNS of mice significantly impaired spatial learning in the Barnes maze test. Compared to Tat− controls, Tat+ mice demonstrated significantly longer latencies to find the escape hole [$F(1,76)=9.39$, $p<0.05$] and spent a significantly lower proportion of time [$F(1,76)=8.17$, $p<0.05$] in the correct quadrant of the Barnes maze (Fig. 1). Irrespective of genotype, all mice took significantly longer to find the escape hole following a 180° swap of the escape location on the probe trial day [$F(4,76)=3.42$, $p<0.05$] compared to any previous day's performance ($p = 0.0001–0.01$; Fig. 1). No differences were observed in the number of errors made or the distance traveled between Tat− and Tat+ mice (Fig. 1); however, a difference in velocity was observed between Tat− and Tat+ mice

[$F(1,76)=5.35$, $p<0.05$] with a significant reduction observed in the reversal probe trial [$F(4,76)=8.45$, $p<0.05$], compared to day 1 performance ($p < 0.0001$). All mice demonstrated significantly decreased maze exploration (indicated by distance traveled) [$F(4,76)=12.86$, $p<0.05$] and made fewer errors [$F(4, 76) = 11.37$, $p < 0.05$] compared to day 1 ($p = 0.0001–0.02$; Fig. 1).

Similarly, HIV-1 Tat expression significantly impaired cognitive performance in a novel object recognition task [$F(1, 26) = 5.67$, $p < 0.05$]. Compared to performance in the training trial, Tat− control mice spent a significantly greater proportion of time with a novel object in the retention trial ($p = 0.0007$; Fig. 2) indicating intact mnemonic performance. However, Tat+ mice demonstrated impaired object recognition during the retention phase (Fig. 2), spending significantly less time investigating the novel object compared to Tat− controls ($p = 0.04$). No significant differences were observed between Tat− or Tat+ mice on the total amount of time spent investigating objects nor on any motor measure investigated (Table 1).

Given that a visual component is present in the Barnes maze and novel object recognition tasks, vision was assessed in a separate group of mice. All Tat− and Tat+ mice responded positively to a stimulus presented to the left and right fields of vision prior to DOX exposure and at 7 and 14 days following DOX exposure.

Hippocampal volume is unchanged following Tat exposure

Point count analysis of hippocampal volume revealed no difference between Tat− (16.71 ± 0.39 mm^3) and Tat+ (17.62 ± 0.47 mm^3) mice. Additionally, the volume of the CA1 subfield was unchanged between Tat− (9.97 ± 0.19 mm^3) and Tat+ (10.74 ± 0.69 mm^3) mice indicating that there was no difference in the volume of the hippocampus or in hippocampal area CA1 following 12–14 days of Tat induction.

HIV-1 Tat expression is associated with reductions in specific CA1 nNOS-expressing interneuron subpopulations

We investigated Tat effects on a subset of nNOS+/NPY+ cells known as ivy cells. These cells are found primarily in the stratum pyramidale as well as the stratum radiatum of CA1 (Fuentealba et al. 2008). To assess the vulnerability of nNOS+/NPY+ cells to HIV-1 Tat, hippocampal sections were immunolabeled for nNOS, NPY, and NeuN (Figs. 3 and 4). The distribution of nNOS and NPY immunoreactivity within CA1 was consistent with previous research (Morris 1989; Jinno et al 1999; Fuentealba et al. 2008). Significant reductions in the percentage of nNOS-expressing cells were observed in the stratum pyramidale [$t(8) = 2.25$, $p < 0.05$] and stratum radiatum [$t(8) = 2.43$, $p < 0.05$] of Tat+ mice compared to Tat− mice (Fig. 3). Importantly, the reduction in nNOS+ interneurons was specifically restricted to the subpopulation of interneurons that did not possess NPY antigenicity in both the stratum pyramidale [$t(8) = 2.36$, $p < 0.05$] and stratum radiatum [$t(8) = 1.92$, $p < 0.05$] after Tat exposure, indicating ivy cells were not selectively vulnerable. No significant differences were observed for NPY+ interneurons lacking nNOS in any layer of CA1. Tat induction did not significantly alter the percentage of NPY+ interneurons or interneurons coexpressing nNOS and NPY in any layer of the hippocampus (Fig. 3).

The proportion of SST+ and PV+ neurons is reduced by HIV-1 Tat expression

To assess the vulnerability of PV+ and SST+ interneurons, hippocampal sections were immunolabeled for PV, SST, and NeuN (Figs. 5 and 6). PV immunoreactivity was nearly exclusive to stratum pyramidale and stratum oriens, consistent with previous research (Kosaka et al. 1987). Observations of SST immunoreactivity in stratum oriens, stratum pyramidale, and stratum radiatum also agree with previous findings (Oliva et al. 2000); however, SST+ neurons occurring in the stratum radiatum were not quantified as part of this study (Fig. 5). The percentage of PV+ neurons were significantly diminished in the stratum pyramidale of Tat+ mice compared to Tat− mice [$t(10) = 1.839$, $p < 0.05$]. While no significant effect of Tat was observed in PV+/SST− interneurons, there was a trend toward significance in PV+/SST+ interneurons in the stratum pyramidale [$t(10) = 1.711$, $p = 0.059$]. No effect of Tat was observed in PV+ interneuron groups in the stratum oriens. In contrast, the proportion of SST+ interneurons was reduced in the stratum oriens [$t(10) = 2.664$, $p < 0.05$], but not in the stratum pyramidale of Tat+ mice. While there were no significant differences in stratum oriens to suggest that subpopulations of SST+ neurons were reduced in the layer, there was a trend toward significant decreases in the proportion of PV−/SST+ [$t(10) = 1.673$, $p = 0.063$]. These data indicate subregion-specific declines in interneuron populations, supporting the idea of selective vulnerability among hippocampal neuron subpopulations.

PV+ and SST+ processes in CA1 appear unaffected by Tat

ImageJ analysis of PVexpression in the stratum radiatum and SST expression in the stratum lacunosum-moleculare showed no differences in fiber density between Tat+ and Tat− mice. There were also no differences in dendritic varicosity number or size in PV+ neurons (Fig. 7).

Discussion

Given the great diversity in function, morphology, and localization of hippocampal interneurons, the highly localized reduction of Syt2+ fibers described by Fitting et al (2013) suggests selective vulnerability of distinct subpopulations of interneurons to HIV-1 Tat. We thus hypothesized that a subpopulation of hippocampal interneurons would be selectively vulnerable to HIV-1 Tat and used an immunohistochemical/morphological approach to identify neurodegenerative changes in cells within defined regions of CA1. We found that Tat+ animals exhibited aberrant learning behavior in both the Barnes maze and novel object recognition tests. Tat expression reduced the population of nNOS+/NPY− interneurons in both the stratum radiatum and stratum pyramidale, SST+ cells in the stratum oriens, and PV + cells in the stratum pyramidale. nNOS+/NPY+ or nNOS−/NPY+ neurons remained unchanged.

The pathology of specific interneuron subpopulations, and the hippocampal interneuron network as a whole, have been demonstrated in a number of neurological disorders (Korotkova et al. 2010; Tóth et al. 2010; Antonucci et al. 2012; Hazra et al. 2013; Levenga et al. 2013), some of which produce behavioral deficits in learning similar to the findings of Fitting et al (2013). Due to neuronal heterogeneity in the hippocampus (Bouilleret et al.

2000; Moga et al. 2002; Avignone et al. 2005), differential responses to Tat-induced excitotoxicity and neuroinflammation may be expected. In a kainate-induced model of excitotoxicity, a loss of GAT-1, a GABA reuptake protein, was greater in the stratum pyramidale than in the stratum oriens and the stratum radiatum, suggesting regional variability in selective vulnerability of hippocampal interneurons (Bouilleret et al. 2000). In HIV models, reductions in the number of cells expressing PV in CA3, and SST in CA1 were observed, although identification of the affected subtypes was incomplete (Masliah et al. 1992; Fox et al. 1997). The results of the present study indicate that there is at least one subtype of nNOS+ interneuron within CA1 that is selectively vulnerable to HIV-1 Tat. nNOS+ interneurons appear in all layers of the hippocampus, a majority of which are subpopulations of neurogliaform cells (NGFCs; nNOS+/NPY−; Jinno et al. 1999; Tricoire et al. 2010). In addition to NGFCs, there are ivy (nNOS+/NPY+) and interneuron-specific interneuron type III (IS3; nNOS+/NPY−) cells. Ivy cells exist primarily in the stratum pyramidale, as well as the stratum radiatum (Fuentealba et al. 2008; Lapray et al. 2012). nNOS+/NPY− cells have been found in the stratum pyramidale (IS3s) and stratum radiatum (NGFCs; Acsády et al. 1996a, b; Porter et al. 1998; Jinno et al. 1999; Jinno and Kosaka 2002, 2004; Tricoire et al. 2010; Armstrong et al. 2012; Somogyi et al. 2012).

Our findings suggest that the ivy cells (i.e., nNOS+/NPY+ neurons residing in the stratum pyramidale) are not vulnerable to HIV-1 Tat as previously hypothesized (Fuentealba et al. 2008). Rather, a significant decline was observed in the number of nNOS+/NPY− interneurons in both the stratum pyramidale and stratum radiatum. In the stratum pyramidale, these vulnerable neurons are thought to be IS3s (given the lack of NPY). Additionally, these neurons are positive for vasoactive intestinal polypeptide (VIP), and calretinin (CR) (Acsády et al. 1996a, b; Porter et al. 1998; Jinno et al. 1999; Jinno and Kosaka 2002; Tricoire et al 2010) and the expression of nNOS differentiates them as IS3s versus an IS1 subtype (Tricoire et al. 2010). Functionally, IS3s support feedback inhibition within CA1 (Gulyás et al. 1996; Chamberland et al. 2010; Chamberland and Topolnik 2012; Tyan et al. 2014). Of particular interest, the selective vulnerability of multiple subsets of CR + cells in the hippocampus may occur in a temporal-lobe epilepsy model, a good predictor of neuronal vulnerability to excitotoxicity. Those cells that remain exhibit pathologic dendritic varicosities and a loss of dendritic complexity and synapses, as a result of excitotoxic injury (Tóth et al. 2010; Tóth and Maglóczky 2014). Patients with epilepsy can have fewer nNOS+ interneurons within CA1 (Leite et al. 2002).

The population of nNOS+/NPY− cells affected by Tat in stratum radiatum likely consists of multiple nNOS+ interneuron subtypes, including IS3s (Acsády et al. 1996a, b; Porter et al. 1998; Jinno et al. 1999; Jinno and Kosaka 2002; Tricoire et al. 2010) and NGFCs (Tricoire et al. 2010; Armstrong et al. 2012; Tricoire and Vitalis 2012; Somogyi et al. 2012). Since IS3s represent a small proportion of nNOS+ cells in the stratum radiatum, it is probable that NGFCs constitute a majority of nNOS+/NPY− cells lost (Jinno et al. 1999; Jinno and Kosaka 2002; Tricoire et al. 2010; Armstrong et al. 2012). The presence or absence of CR, which is expressed in IS3s, but not NGFCs, might be used to further differentiate these interneuron subtypes in the future (Jinno and Kosaka 2002; Armstrong et al. 2012; Somogyi et al. 2012). Considering the lack of cell death in the hippocampus observed at early time points following Tat induction (Fitting et al. 2013), we speculate that the affected

interneuron subtypes are downregulating nNOS in response to excitotoxic injury in order to restore homeostasis (Hu et al. 2008; Di et al. 2012).

nNOS is required for the normal functioning of the hippocampal interneuron network and maintenance of related behavioral endpoints. In complete nNOS knockout mice, there is an attenuation of contextual fear conditioning and spatial memory formation similar to what is observed in Tat transgenic mice (Kirchner et al. 2004; Weitzdoerfer et al. 2004; Kelley et al. 2009; Fitting et al. 2013). While nNOS is important for normal hippocampal function (Kirchner et al. 2004; Zanelli et al. 2009), research indicates that nNOS-induced neuronal damage occurs in pathological states (Cui et al. 2007; Eugenin et al. 2007; Steinert et al. 2010; Wang et al. 2010; Drury et al. 2014; Hsu et al. 2014; Wu et al. 2014). The role of nNOS in excitotoxicity, including HIV-1 Tat excitotoxicity, is reportedly linked to its activation of PSD-95 and NMDA receptor complexes (Christopherson et al. 1999; Eugenin et al. 2007; Fan et al. 2010; King et al. 2010; Steinert et al. 2010). Exposure to excitotoxic levels of glutamate causes temporal dysregulation of nNOS activation-inactivation processes (Rameau et al. 2007). Thus, Tat-induced increases in the activation of nNOS may enhance NMDA receptor phosphorylation, the recruitment of additional AMPA receptors, and the generation of peroxynitrite resulting in greater susceptibility to excitotoxic damage in nNOS-expressing interneurons (Yu et al. 1997; Grima et al. 2001; Rameau et al. 2007; Hossain et al. 2012). Lastly, we speculate that the proposed compensatory downregulation of nNOS may be absent or may be less prevalent in nNOS+/NPY+ neurons due to the neuroprotective effects of NPY, which can regulate neuronal excitability via paracrine/autocrine signaling (Xapelli et al. 2006; Smialowska et al. 2009).

The present findings stand in contrast to prior studies that did not demonstrate a loss of PV+ interneurons in CA1 of HIV-infected patients or rodent models (Masliah et al. 1992; Guo et al. 2012). Herein, we differentiate the percentages of CA1 PV-expressing cells by CA1 layer and by SSTco-expression, allowing for delineation of CA1 cell types (PV+ interneurons including bistratified and oriens-lacunosum-moleculare (O-LM) cells; Kosaka et al. 1987; Buhl et al. 1994; Masliah et al. 1992; Freund and Buzsáki 1996; Jinno and Kosaka 2000, 2002a; Klausberger et al. 2004; Klausberger and Somogyi 2008; Müller and Remy 2014; Yamada and Jinno 2015). Bistratified cells were discerned from O-LM interneurons via the relative intensity of PV (high in bistratified) and SST (minimal in bistratified) immunoreactivity. In contrast, O-LMs exert an opposing PV/SST profile, with a subset of O-LMs being entirely PV− (Chittajallu et al. 2013; Müller and Remy 2014). Our data show a modest effect of Tat on the total PV+ population of the stratum pyramidale; however, this was not restricted to a particular PV+ cell type. Although we believe Tat may be selectively affecting bistratified cells based on a trend toward reduced PV+/SST+ interneuron numbers, additional information is needed for confirmation. Interestingly, while the loss of PV+ cells in CA1 is not reported in patients with neuroAIDS, many of the PV+ interneurons were reportedly unhealthy with damaged neurites being the chief descriptor (Masliah et al. 1992). Despite limitations in generalizing findings from Tat transgenic mice to HIV-infected individuals, the collective findings suggest PV+ interneuron subsets are selectively vulnerable to HIV—perhaps through synaptodendritic injury and pruning.

Reductions in SST gene expression in the brains of HIV patients are linked to the development of depression (Everall et al. 2006). Fox et al. (1997) showed a reduction in SST + interneurons in HIV patients, but did not differentiate between the layers of CA1. We observed a strong net reduction of SST+ interneurons in the stratum oriens, but not in the stratum pyramidale. The stratum radiatum was not quantified due to the relative rarity of SST+ cells in this region. Interestingly, there was a trend toward reductions in SST+/PV− interneurons in the stratum oriens following Tat induction. The loss of SST has been shown to have profound effects on LTP within CA1, with differential effects in the apical and basilar dendrites of pyramidal cells based on receptor subtypes and response to specific oscillation patterns (Fan and Fu 2014). Considering the decline in SST+ neurons observed in this study and previous findings of reduced SST expression in HIV patients (Everall et al. 2006), it will be important to examine whether region-specific SST losses contribute to impairments in LTP and related behavior following Tat-exposure (Behnisch et al. 2004; Fitting et al. 2013). In support of this notion, ablating SST+ interneurons, focal to distal apical dendrites of pyramidal cells in SLM (likely O-LMs), causes a loss of cue-based fear-learning, while removal of PV+ neurons, focal to basal dendrites and proximal portions of apical dendrites in the stratum radiatum, does not result in loss of fear learning (Leão et al. 2012; Lovett-Barron et al. 2014; Müller and Remy 2014). While it is not possible to say with certainty which SST+ population is affected in the present study, the net loss in SST in the stratum oriens is most likely due to O-LM cell vulnerability.

We and others have found that HIV-1 Tat can impair learning and memory, and the present study begins to advance our understanding of the limbic neuronal types that may underlie such effects. In the present study, Tat+ mice exhibited deficient performance on two hippocampally dependent tasks, the Barnes maze and novel object recognition tests, compared to their Tat− counterparts. Consistent with these results, stereotaxic injections of Tat directly to dorsal hippocampus have recently been demonstrated to increase the latency to find a hidden platform in a Morris water maze and to impair novel object recognition among Sprague-Dawley rats (Harricharan et al. 2015). Similar intracerebroventricular injections of Tat impaired radial arm maze performance concurrent with disrupted LTP in the hippocampus of C57BL/6J mice (Li et al. 2004). Additionally, similar impairment of Barnes maze performance and object recognition has been observed in a separate GFAP-driven Tat-inducible mouse model (Carey et al. 2012). Considering that behavioral deficits similar to those seen in neuroAIDS can be traced back to the dysfunction of one or more of the interneuron types discussed here, it is reasonable to assume that imbalance in this complex circuit may contribute to the attenuation of spatial memory observed in HAND (Masliah et al. 1992; Buhl et al. 1994; Sik et al. 1995; Acsády et al. 1996b; Fox et al. 1997; Ali et al. 1998; Kirchner et al. 2004; Klausberger et al. 2004; Weitzdoerfer et al. 2004; Carey et al. 2012; Leão et al. 2012; Fitting et al. 2013; Lovett-Barron et al. 2014; Sun et al. 2014; Tyan et al. 2014; Müller and Remy 2014).

Hippocampal interneurons interact as part of a functional network and it is clear that the removal of even one cell type from the network can have drastic effects on information processing, pyramidal cell excitation, and consequent behavioral outcomes (Moga et al. 2002; Dugladze et al. 2007; Goldin et al. 2007; Tóth et al. 2010; Peng et al. 2013; Long et al. 2014; Lovett-Barron et al. 2014; Lovett-Barron and Losonczy 2014; Tóth and Maglóczky

2014; Orbán-Kris et al. 2015). Importantly, the susceptible nNOS+/NPY− interneurons of the stratum pyramidale and the stratum radiatum, PV+ cells of the stratum pyramidale, and SST+ interneurons of the stratum oriens form a microcircuit known to be involved in a complex feedback loop/input gating mechanism that regulates network synchronization within CA1 (Fig. 8; Buhl et al. 1994; Sik et al. 1995; Acsády et al. 1996b; Ali et al. 1998; Klausberger et al. 2004; Chamberland et al. 2010; Chamberland and Topolnik 2012; Leão et al. 2012; Sun et al. 2014; Tyan et al. 2014; Müller and Remy 2014; Milstein et al. 2015). Herein, we propose this microcircuit to be selectively vulnerable to HIV-1 Tat. Given clinical observations that implicate HAND- and psychostimulant-related neurocognitive deficits to be associated with interneuron losses in other brain regions (Chana et al. 2006), the present findings suggest that a proportionally small, interconnected ensemble of hippocampal CA1 interneurons may contribute to key functional and neurobehavioral deficits observed in HAND.

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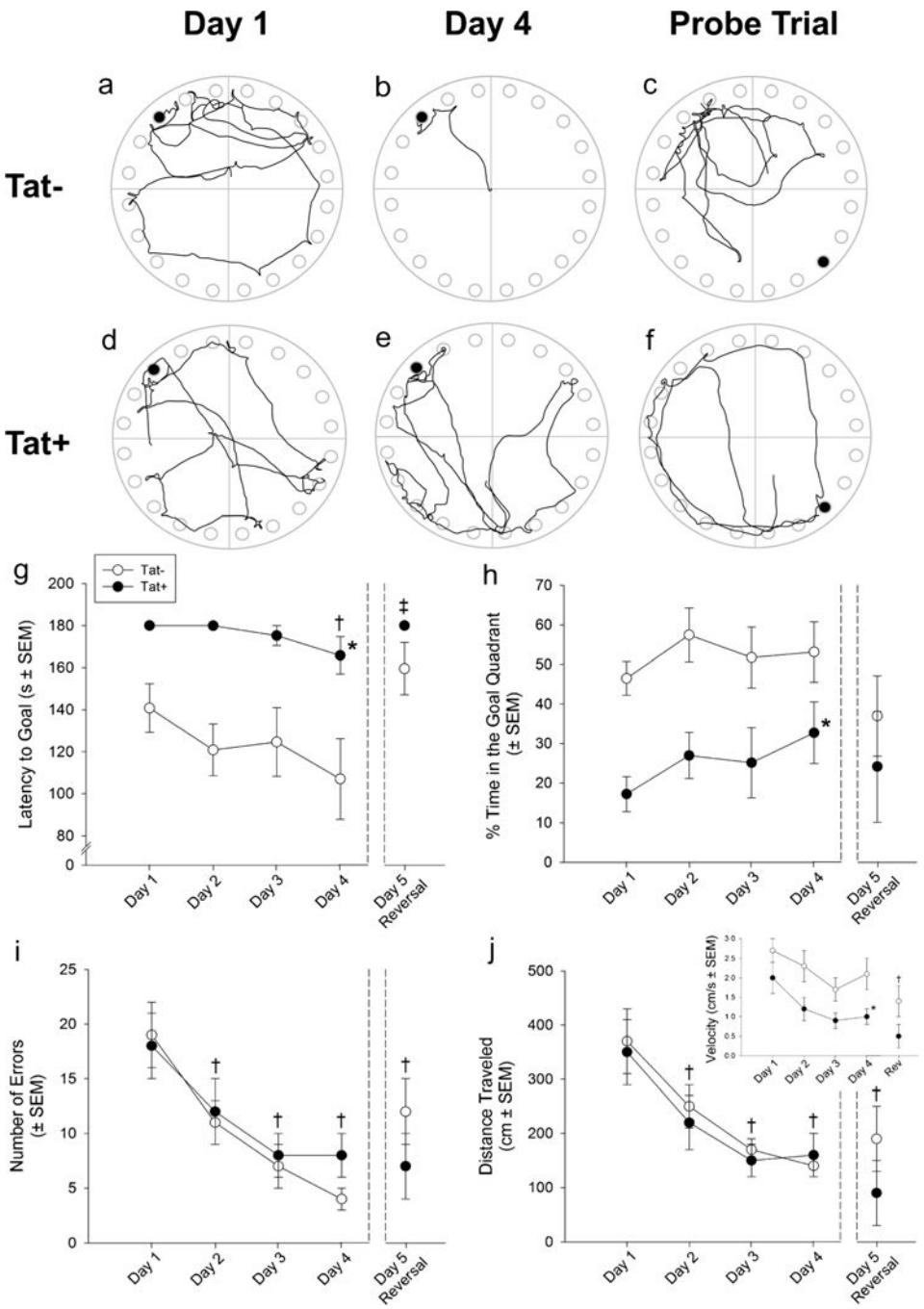

Fig. 1.

Representative traces of the movements of Tat− (**a**–**c**) and Tat+„ (**d**–**f**) mice exploring a Barnes maze on testing days 1 (**a**, **d**) and 4 (**b**, **e**), as well as the reversal probe trial [escape hole reversed 180° (**c, f**)]. Escape hole is noted as the *black circle* in each figure. Tat+ mice (n = 8) demonstrate significantly longer latencies to find the escape hole (**g**) and (**h**) spend a significantly lower proportion of time in the correct quadrant of the Barnes maze test, indicating impaired learning compared to Tat− control mice (n = 13). Neither the number of errors made **(i**) nor the distance traveled (**j**) differs between Tat− and Tat+ mice, but

decreases among all animals from day 1 performance. Tat+ mice have a reduced velocity compared to Tat− mice, and all mice show reduced velocity in the reversal probe trail compared to day 1 performance (**j**, *inset*). The *asterisk* indicates a main effect for Tat− and Tat+ mice to significantly differ. The *dagger sign* indicates performance significantly differs from day 1 performance. The *double dagger* sign indicates reversal probe trial performance significantly differs from all other testing days, except day 1 (main effects, repeated measures ANOVA, $p < 0.05$)

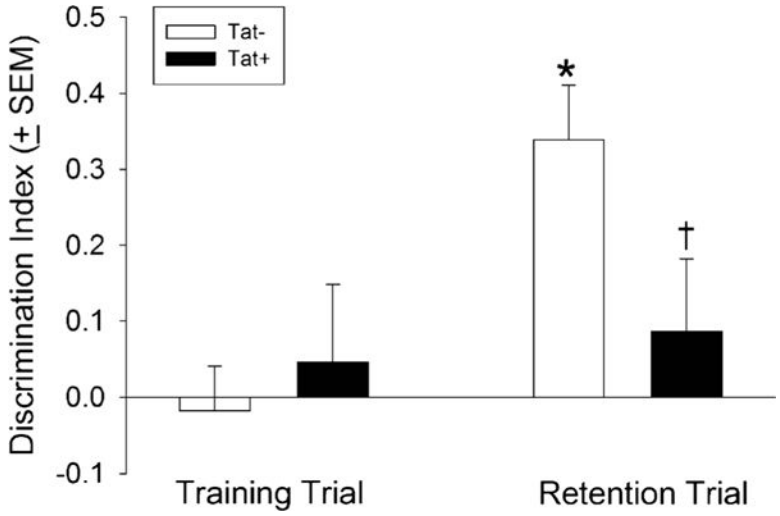

Fig. 2.

Tat+ mice ($n = 14$) spend a significantly lower proportion of time investigating a novel (vs. familiar) object in a novel object recognition test, indicating impaired cognitive performance compared to Tat− control mice ($n = 14$). The *asterisk* indicates a significant difference from the respective training trial exploration. The *dagger sign* indicates a significant difference between Tat− and Tat+ mice in the retention trial (interaction, repeated measures ANOVA, $p < 0.05$)

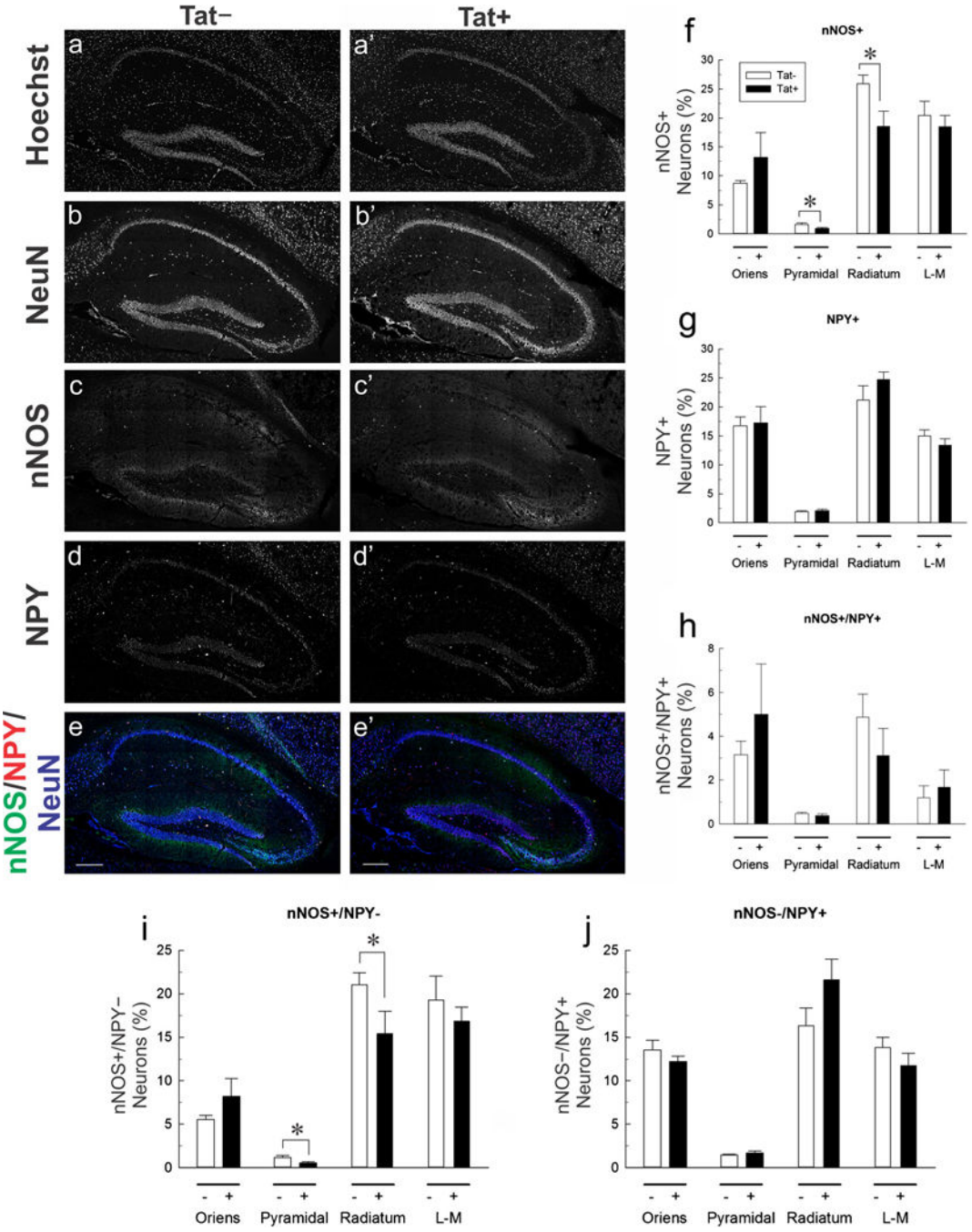

Fig. 3.

Effects of HIV-1 Tat induction on neuronal nitric oxide synthase (nNOS) and neuropeptide Y (NPY) immunoreactive interneuron subpopulations in hippocampal area CA1. **a**–**e** Immunoreactivity for nNOS (**c, c′**), NPY (**d**, **d′**), and the neuronal marker, NeuN (**b, b′**), was colocalized in sections from Tat+ and Tat− mice counterstained using Hoechst dye (**a, a′**). **e, e′** NeuN (*blue*), nNOS (*green*), and NPY (*red*) merged. **f**–**j** The percentage of labeled neurons in hippocampal area CA1 by layer and marker. The proportion of nNOS+ neurons in stratum pyramidale (Pyramidal) and stratum radiatum (Radiatium) is significantly

decreased in Tat+ compared to Tat− mice (*$p < 0.05$, one tailed t test) (**f**), while the percentage of NPY+ neurons or neurons co-expressing nNOS and NPY in any layer is unaffected by Tat (**g**, **h**). The percentage of cells expressing nNOS in the absence of NPY is significantly reduced in stratum pyramidale and in stratum radiatum (*$p < 0.05$, one tailed t test) (**i**), while the proportion of NPY cells that lack nNOS was unaffected by Tat (**j**). Stratum oriens (Oriens), stratum lacunosum-moleculare (L–M). *Scale bar* = 200 μm

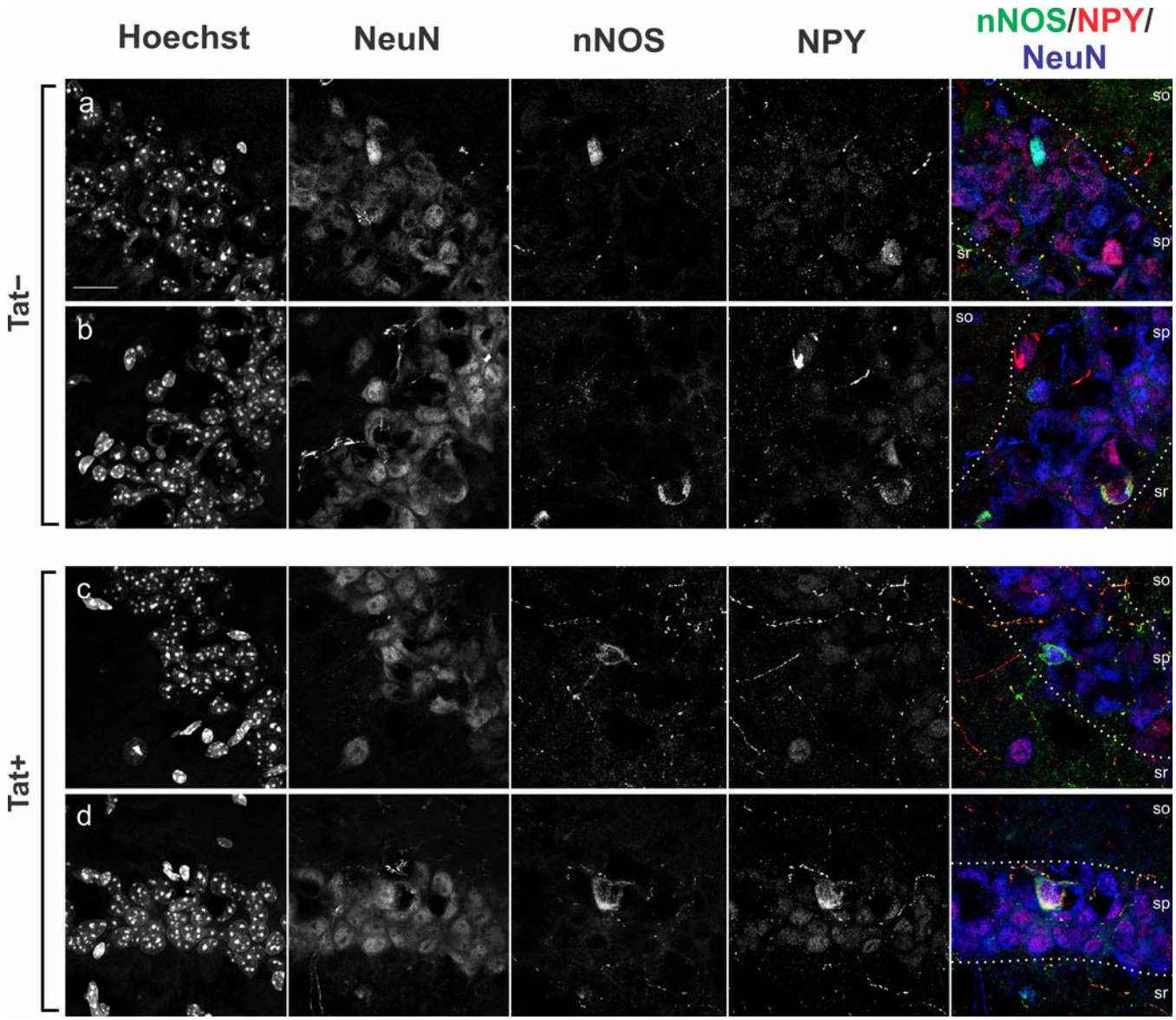

Fig. 4.

Subcellular localization of neuronal nitric oxide synthase (nNOS) and neuropeptide Y (NPY) immunoreactivity in interneurons in hippocampal area CA1 of Tat+ and Tat− mice. **a**–**d** Immunoreactivity for nNOS (*green*), NPY (*red*), and the neuronal marker, NeuN (*blue*), was colocalized in sections from Tat+ and Tat− mice counterstained using Hoechst dye (nuclei). **a**, **c** nNOS+/NPY− interneurons located in stratum pyramidale (sp). **b**, **d** nNOS+ and NPY+ interneurons of stratum pyramidale. Stratum oriens (so), stratum radiatum (sr). *Scale bar* = 10 μm

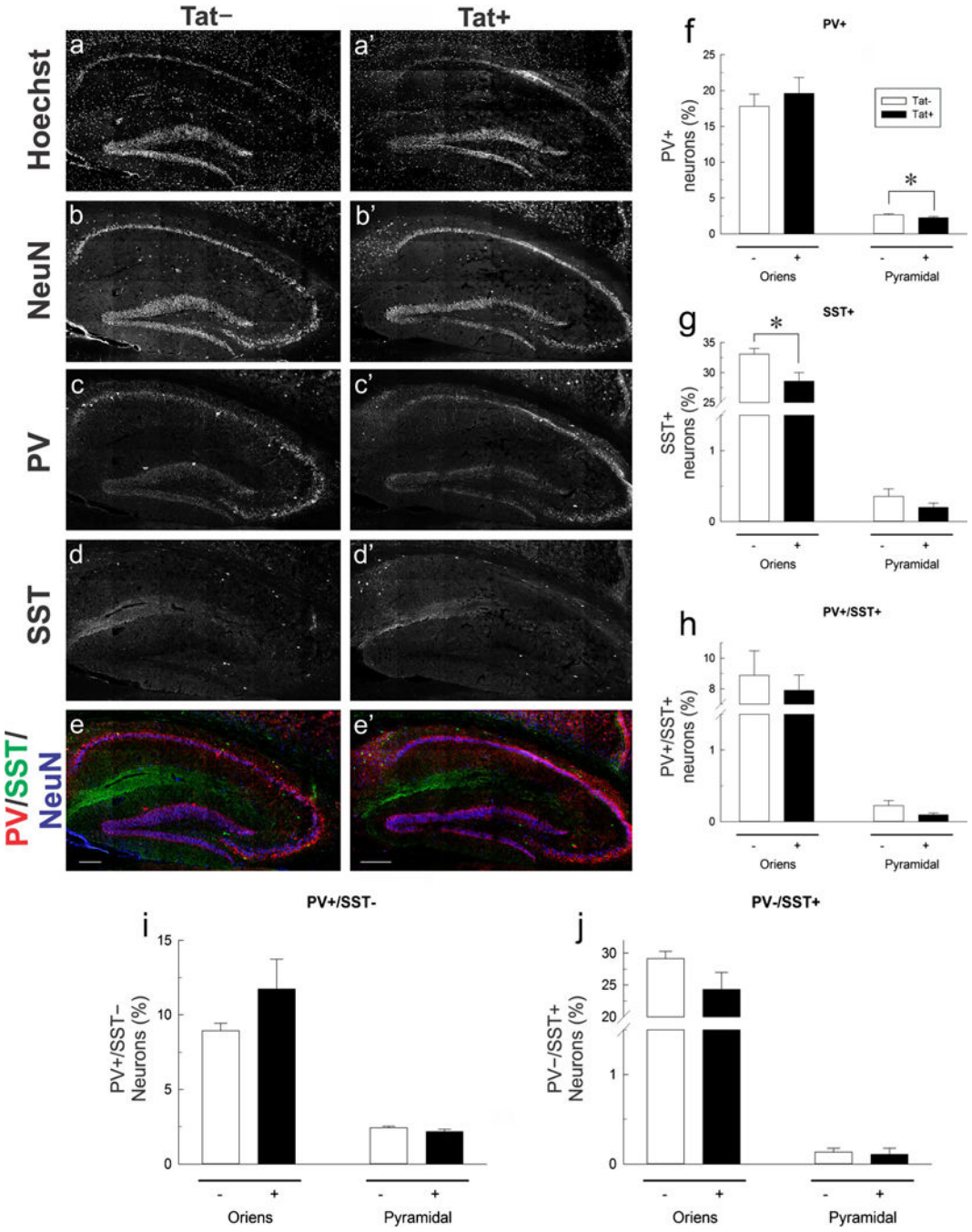

Fig. 5.

Effects of HIV-1 Tat induction on parvalbumin (PV) and somatostatin (SST) immunoreactivity in interneurons within hippocampal area CA1. **a**–**d** Low-magnification images of PV, SST, and NeuN immunoreactivity in sections from Tat+ and Tat− mice additionally counterstained with Hoechst dye. **a**–**e** Hippocampal sections from Tat+ and Tat– mice treated with DOX, probed with Hoechst (**a**, **a**′), NeuN (**b**, **b**′), PV (**c**, **c**′), and SST (**d**, **d**′). **e**, **e**′ NeuN (*blue*), PV (*green*), and SST (*red*) merged. **f**–**j** The percentage of labeled neurons in hippocampal area CA1 by layer and marker. A decrease in the percent of PV+

neurons is seen in stratum pyramidale (Pyramidal) (**f**), and a decrease in the percent of SST+ neurons is observed in stratum oriens (Oriens) (**g**). No significant decrease is observed for neurons expressing both PV and SST or for neurons that express either marker in the absence of the other (**h**–**j**). The *asterisk* denotes a significant reduction from Tat− control population percentage (one tailed *t* test, $p < 0.05$). *Scale bar* = 200 μm

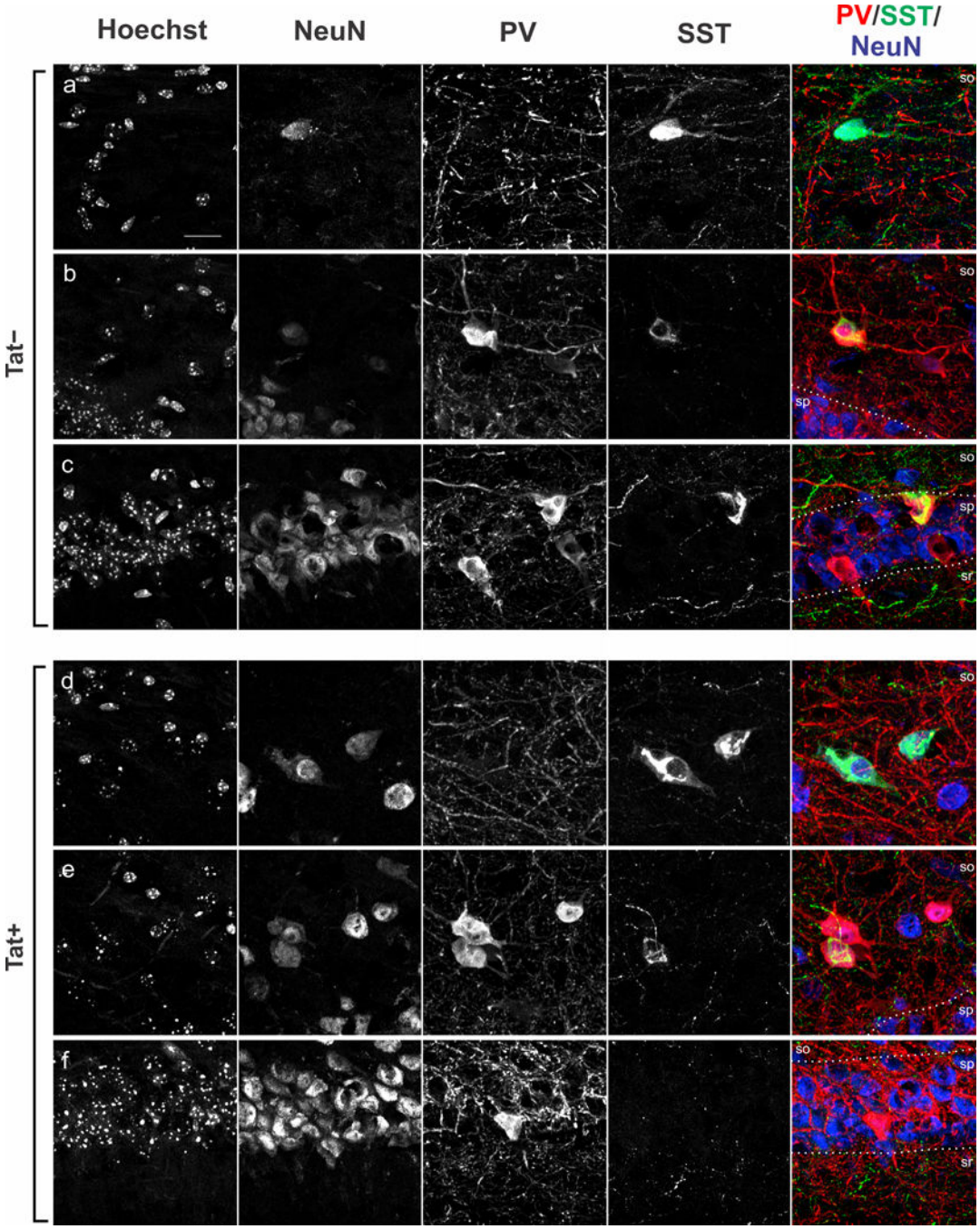

Fig. 6.

Subcellular localization of parvalbumin (PV) and somatostatin (SST) immunoreactivity in interneurons in hippocampal area CA1 of Tat+ and Tat− mice. **a**–**d** High-magnification images of PV (*red*), SST (*green*), and NeuN (*blue*) immunoreactivity in sections from Tat+ and Tat− mice additionally counterstained with Hoechst dye (**a**–**f**). **a**, **d** PV+/SST− interneurons located in stratum oriens (so). **b**, **e** PV+ and SST+ interneurons of the stratum oriens. **c**, **f** PV+ neurons of stratum pyramidale (sp). Stratum radiatum (sr). *Scale bar* = 10 μm

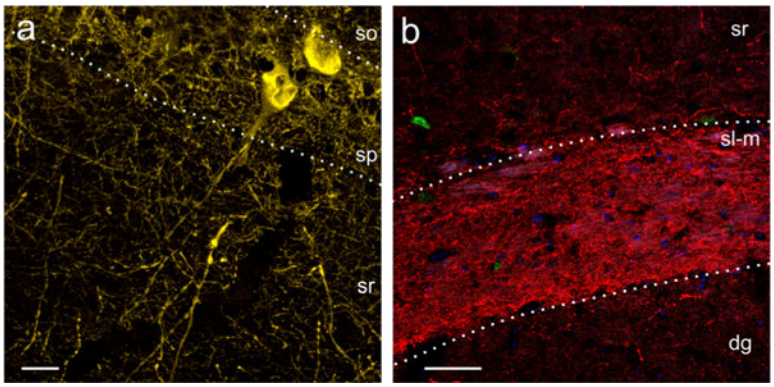

Fig. 7.

No evidence of changes to parvalbumin-immunoreactive (PV+) dendrites or somatostatin-immunoreactive (SST+) axons is observed following Tat induction. **a** PV+ neurons and neurites in Tat− mice. No differences in the density of PV+ fibers or increases in the number of dendritic varicosities was seen in the stratum radiatum (sr). *Scale bar* = 20 μm. **b** Tat− SST+ axons in stratum lacunosum-moleculare (sl-m). No difference in fiber density of SST+ axons in the SL-M was observed. Stratum oriens (so), stratum pyramidale (sp), dentate gyrus (dg). *Scale bar* = 50 μm

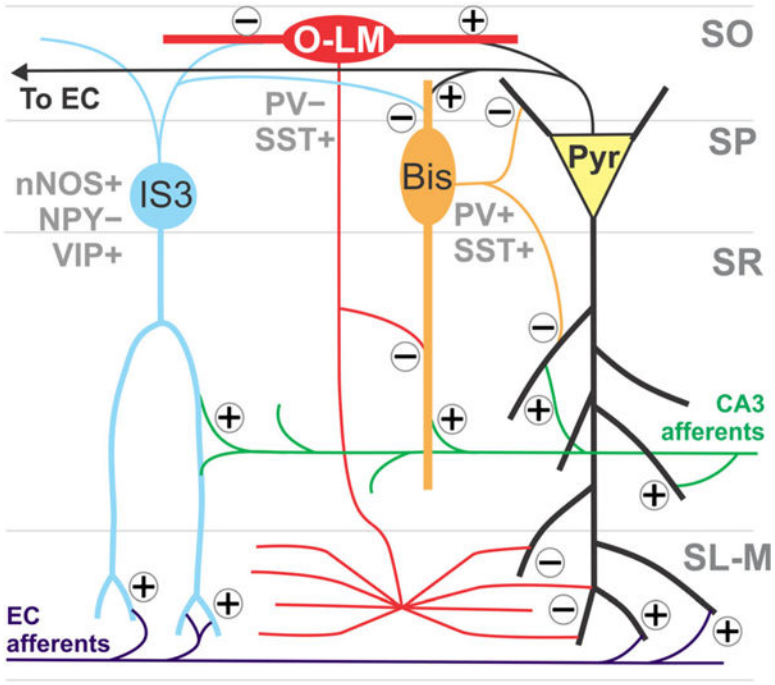

Fig. 8.

The interneurons affected by Tat form a microcircuit within the CA1 area of the hippocampus. CA1 pyramidal cells receive inputs from both hippocampal area CA3 and the entorhinal cortex. Oriens-lacunosum-moleculare (O-LM) cells gate inputs from the entorhinal cortex onto pyramidal cell distal apical dendrites. CA3 inputs are disinhibited by bistratified cells and Shaffer collateral-associated interneurons in the stratum radiatum (Sik et al. 1995; Klausberger et al. 2004; Leão et al. 2012). O-LMs receive excitatory inputs from CA1 pyramidal cells and the septum, and inhibitory innervation from interneuron-specific interneuron type 3 (IS3) cells (Acsády et al. 1996b; Sun et al. 2014; Tyan et al. 2014; Müller and Remy 2014). Bistratified cells receive excitatory input from Shaffer collaterals, CA1 pyramidal cells, and the septum (Ali et al. 1998; Klausberger et al. 2004; Müller and Remy 2014) and innervate pyramidal cells (Buhl et al. 1994; Sik et al. 1995; Klausberger et al. 2004). In addition to being inhibited by O-LMs, bistratified cells receive GABAergic inputs from the neuronal nitric oxide (nNOS)+/neuropeptide Y (NPY)− IS3s (Leão et al. 2012; Tyan et al 2014). IS3s innervate O-LMs preferentially, as well as bistratified cells, in the stratum oriens (Acsády et al. 1996b; Tyan et al. 2014). Disruption of this circuit by Tat may disrupt the gating of inputs from CA3 and the entorhinal cortex and therefore produce aberrant pyramidal cell outputs, which could account for the behavioral phenotype observed following Tat exposure (Sik et al. 1995; Klausberger et al. 2004; Leão et al. 2012). CA1 pyramidal cell (*yellow*), bistratified cell (Bis, *orange*), O-LM (red), IS3 (light *blue*), Shaffer collateral input from CA3 (*green*), perforant path input from the entorhinal cortex (EC, *purple*). Glutamatergic synapses are noted with a *plus* (+) and GABAergic synapses are noted with a *minus* (−)

Table 1

Raw exploration time and motor behavior among Tat− and Tat+ mice (n = 14/group) that were assessed in a novel object recognition task (mean ± SEM)

	Tat−, *n* = 14	**Tat+, *n* = 14**
Familiar object exploration (s)	12 ± 2	15 ± 2
Novel object exploration (s)	24 ± 3	20 ± 3
Total distance traveled (m)	13 ± 1	11 ± 1
Rearing events per trial (10 min)	51 ± 8	44 ± 8
Total time rearing (s)	43 ± 7	35 ± 6

No significant differences were observed between Tat− and Tat+ mice