



HHS Public Access

Author manuscript

J Neurochem. Author manuscript; available in PMC 2020 April 01.

Published in final edited form as:

J Neurochem. 2019 April ; 149(1): 98–110. doi:10.1111/jnc.14668.

Effects of HIV-1 Tat on oligodendrocyte viability are mediated by CaMKII β -GSK3 β interaction

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Abstract

Myelin disruptions are frequently reported in human immunodeficiency virus (HIV)-infected individuals and can occur in the CNS very early in the disease process. Immature oligodendrocytes (OLs) are quite sensitive to toxic increases in [Ca²⁺]_i caused by exposure to HIV-1 Tat (transactivator of transcription, a protein essential for HIV replication and gene expression), but sensitivity to Tat-induced [Ca²⁺]_i is reduced in mature OLs. Tat exposure also increased the activity of Ca²⁺/calmodulin-dependent kinase II β (CaMKII β), the major isoform of CaMKII expressed by OLs, in both immature and mature OLs. Since CaMKII β is reported to interact with glycogen synthase kinase 3 β (GSK3 β), and GSK3 β activity is implicated in OL apoptosis as well as HIV neuropathology, we hypothesized that disparate effects of Tat on OL viability with maturity might be due to an altered balance of CaMKII β -GSK3 β activities. Tat expression *in vivo* led to increased CaMKII β and GSK3 β activity in multiple brain regions *in transgenic mice*. *In vitro*, immature *murine* OLs expressed higher levels of GSK3 β , but much lower levels of CaMKII β , than did mature OLs. Exogenous Tat upregulated GSK3 β activity in immature, but not mature, OLs. Tat-induced death of immature OLs was rescued by the GSK3 β inhibitors valproic acid or SB415286, supporting involvement of GSK3 β signaling. Pharmacologically inhibiting CaMKII β increased GSK3 β activity in Tat-treated OLs, and genetically knocking down CaMKII β promoted death in mature OL cultures treated with Tat. Together, these results suggest that the effects of Tat on OL viability are dependent on CaMKII β -GSK3 β interactions, and that increasing CaMKII β activity is a potential approach for limiting OL/myelin injury with HIV infection.

SUMMARIZING SCHEMATIC

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Conflict of interest: The authors declare no competing financial interests. *B.Fuss* is a former editor of *The Journal of Neurochemistry* and an ISN council member.

The HIV-1 protein Tat (transactivator of transcription) is documented to cause significant neurotoxicity. We have reported that immature oligodendrocytes (OLs) are also quite vulnerable to toxic effects of HIV-1 Tat, through Ca^{2+} -mediated mechanisms. More mature OLs experience similarly elevated $[\text{Ca}^{2+}]_i$ but display significantly less cytotoxicity. Based on findings presented here, we propose that this differential toxicity may reflect changes in the relative levels of GSK3 β versus CaMKII β as OLs mature. HIV-1 Tat-dependent increases in $[\text{Ca}^{2+}]_i$ activate both GSK3 β and CaMKII β . In mature OLs, large amounts of cytoplasmic CaMKII β are activated to inhibit GSK3 β , protecting OLs from death. Conversely, lower levels of CaMKII β in immature OLs are insufficient to protect against the high levels of activated GSK3 β . This relative overactivation of GSK3 β triggers signaling events leading to immature OL death. The findings support the concept that increasing CaMKII β levels/activity may mitigate $[\text{Ca}^{2+}]_i$ -induced toxicity in multiple injury/disease situations. Grey bars represent the relative activity levels in OLs at different levels of maturity and activation.

Keywords

neuroAIDS; cell death; myelination; calcium; hippocampus; striatum; cortex

INTRODUCTION

Infiltration of human immunodeficiency virus (HIV) into the central nervous system (CNS) occurs at an early, asymptomatic stage of the disease process. Despite effective combined antiretroviral therapy (cART), ~50% of HIV⁺ individuals still suffer a variety of mild neuronal deficits, collectively called HIV-associated neurocognitive disorders (HAND) (Sacktor *et al.* 2001). White matter tracts are commonly affected in HAND patients (Gorry *et al.* 2003). In the white matter of post-mortem HIV tissues, oligodendrocytes (OLs) exhibit up-regulated p53 and BAX (Jayadev *et al.* 2007), indicating activation of cell death signaling. OLs are unlikely to be directly infected by HIV given their lack of CD4 expression (Sattentau *et al.* 1986) and the typically low viral load in the CNS, especially following cART (Yilmaz *et al.* 2006). Although HIV virions have been observed in OLs by electron microscopy (Gyorkey *et al.* 1987), and HIV has been proposed to infect OLs using galactosylceramide on the cell surface as alternative receptors (Albright *et al.* 1996), those reports remain quite controversial. Thus, OL damage is more likely to be caused by glial inflammatory responses or toxicity from secreted viral proteins. Although infection- or virotoxin-induced inflammation is thought to be the main cause of HAND pathogenesis, the extent to which adjunctive anti-inflammatory drug therapy improves neurocognitive scores in HIV⁺ individuals maintained on cART is debated, suggesting that additional injury mechanisms may be operative (Sacktor *et al.* 2011, Tan & McArthur 2012, McArthur *et al.* 2010).

The HIV-1 transactivator of transcription (Tat) is a highly conserved protein, which is essential to HIV replication and gene expression (Dayton *et al.* 1986, Fisher *et al.* 1986). Expression of Tat starts early during HIV infection and continues through the viral lifespan, even during cART-mediated suppression of viral replication (Bagashev & Sawaya 2013, Heaton *et al.* 2010, Johnson & Nath 2016). *Significant quantities of Tat are released from*

infected cells, and extracellular or exogenous Tat can be actively internalized by (Ensoli *et al.* 1993, Frankel & Pabo 1988), or palmitoylated within the cell membrane (Chopard *et al.* 2018) of different bystander cells types, and can modulate host gene expression and cellular functions, including cell survival and development. Previously, we reported that Tat activates OL surface ionotropic glutamate receptors (iGluRs), resulting in elevated intracellular calcium ($[Ca^{2+}]_i$) and Ca^{2+} /Calmodulin dependent kinase II β (CaMKII β) activity. Different cell death responses in immature and mature OLs (Zou *et al.* 2015) suggested activation of divergent downstream signaling pathways. We have been intrigued by the potential relationship between CaMKII β and glycogen synthase kinase 3 β (GSK3 β), one of the two isoforms of GSK3 that was originally discovered as a serine/threonine kinase vital for glucose metabolism. Inhibiting GSK3 β activity *in vivo* can promote OL precursor cell proliferation, OL survival, and subsequent myelination (Azim & Butt 2011), indicating its involvement in OL development and function. Song *et al.* (2010) showed that inhibitory phosphorylation of GSK3, especially GSK3 β at its N-terminal serine residue by CaMKII, promotes survival of cerebellar granule neurons. Interestingly, the major isoform of CaMKII in cerebellar granule neurons, CaMKII β , is also the predominant CaMKII in OLs (Burgin *et al.* 1990, Waggener *et al.* 2013), and CaMKII β is critical for OL maturation under physiological conditions (Waggener *et al.* 2013).

Since the activities of GSK3 β and CaMKII β can both be regulated by increased $[Ca^{2+}]_i$ (Hartigan & Johnson 1999, Hudmon & Schulman 2002b), we hypothesized that a difference in the balance of GSK3 β and CaMKII β levels in immature and mature OLs may contribute to divergent responses following Tat-driven $[Ca^{2+}]_i$ increases. Here we report that Tat expression *in vivo* in the adult brain led to increased activity of both CaMKII β and GSK3 β in multiple regions. Tat also increased GSK3 β activity in immature OLs *in vitro*; this increase in GSK3 β activity was accompanied by a decrease in survival, which was rescued by the GSK3 β inhibitors, valproic acid (VPA) or SB415286. Conversely, mature OLs expressed relatively low basal levels of GSK3 β compared to immature OLs, and Tat did not increase their GSK3 β activity or alter their survival. Tat activated OL CaMKII β at both developmental stages, although mature OLs had significantly higher basal levels of CaMKII β . Inhibiting CaMKII β pharmacologically increased GSK3 β activity and genetically knocking down CaMKII β made mature OL cultures vulnerable to Tat. Thus it appears that mature OLs are protected against Tat toxicity by a combination of high basal levels of CaMKII β , low basal levels of GSK3 β activity, and failure of Tat to increase GSK3 β activity.

Together, these results suggest that Tat effects on OL viability reflect a balance of CaMKII β -GSK3 β signaling, and infer that therapeutic approaches to alter activity in these pathways to favor CaMKII β might protect against OL/myelin deficits in HIV⁺ individuals, perhaps delaying the appearance of HAND.

MATERIALS AND METHODS

Experiments involving animals were carried out strictly in compliance with procedures reviewed and approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (Approval AM10158). All mice were housed 3–5 per cage with littermates in the Department of Animal Resources vivarium, in a temperature and humidity-

controlled room on a 12 h light-dark cycle (lights off 18:00), with *ad libitum* access to food and chow. *Anti-nociceptive medication was not required by any animal in these studies. The studies were not pre-registered. For experiments presented in Fig. 2 animals were sorted into groups based on their genotype and could not be randomized. In all other studies, animals were arbitrarily selected from among litters of like animals. Analyses in Fig. 2 were performed in a blinded manner using eartag identification numbers.*

Transgenic Mice

Transgenic mice carrying a doxycycline (DOX)-inducible, human glial fibrillary acidic protein-driven Tat expression vector were developed by Dr. Avindra Nath, bred in our facility and utilized as previously described in multiple publications from our lab and others (Zou et al. 2015). The original C3H x C57Bl/6J line has been repeatedly backcrossed to C57Bl/6J. Three-month old male mice (4 Tat⁻ and 4 Tat⁺; 25–30 g; genotyped by PCR and identified by eartag) were given *ad libitum* access to DOX-containing chow (6 g/kg) for 10 d to induce Tat expression, then humanely euthanized by exposure to isofluorane followed by cervical dislocation. Frontal cortex, cerebellum, striatum, and hippocampus were dissected on ice and stored in –80°C before proteins were extracted for Western blotting. *Camk2b^{fl/fl}* mice carrying a targeted and loxP-modified *Camk2b* allele were produced in Dr. Babette Fuss's lab. The mice were generated from strain *Camk2b^{tm1a}(EUCOMM)H^{gmu}* obtained from the European Mouse Mutant Archive (RRID:IMSR_EM:05782). Re-derivation of embryos and initial breeding was done by the Transgenic Mouse Facility at VCU. The Cre-mediated excision of the loxP-flanked exon 2 of the *Camk2b* gene was achieved by adding cell-permeable Cre recombinase (50 nM, #EG-1025, Excellgen, Rockville, MD) to culture media. *Both transgenic lines are available upon reasonable request.*

Oligodendroglial cultures

Mixed glial cultures from postnatal day 0–1 CD-1 (RRID:IMSR_CRL:22, Charles River Laboratory, Wilmington, MA) or *Camk2b^{fl/fl}* mice were prepared as described previously (Zou et al. 2011). Brains were dissected *after decapitation*, minced, and mixed with 2.5 mg/ml trypsin (#T9935, Sigma, St. Louis, MO) and 0.015 mg/ml DNase (#D5025, Sigma) in Dulbecco's Modified Eagle's Medium (DMEM) (#11995–065, Life Technologies, Carlsbad, CA). Dissociated tissues were incubated for 30 min (37°C, 5% CO₂), triturated, and re-suspended in DMEM supplemented with fetal bovine serum (10%, #SH3007003HI, Thermo Scientific HyClone, Logan, UT), glucose (6 g/L, #G7021, Sigma), sodium bicarbonate (#25080–094, 3.7 g/L, Life Technologies), and penicillin/streptomycin (#15070063, Life Technologies). Cells were filtered sequentially through 100 µm (#542000) and 40 µm (#542040) EASYstrainer sieves (Greiner Bio-One, Monroe, NC) and plated in poly-L-lysine-coated (1 mg/ml, #P2636, Sigma) flasks (T25, Corning Inc., Corning, NY) at a density of 2 brains/flask. Eight days later, flasks were rotated at 100 rpm for 20 min to dislodge loosely-attached microglia. The medium was then replaced with DMEM containing 5% fetal bovine serum, and flasks were stroked against the table 5–10 times to release O2A/glia progenitor cells. The resulting cell suspension was panned on a plastic, uncoated culture dish for 2 h to allow adherent glia to attach. Progenitors in the suspension were spun down and plated on poly-L-lysine-coated surfaces in DMEM supplemented with CNTF (10

ng/ml, #450-13, Peprotech, Rocky Hill, NJ), NAC (5 µg/ml, #A9165, Sigma), and triiodothyronine (15 nM, #T6397, Sigma). Culture medium was changed every other day.

Viral protein and drug treatments

OLs were treated with HIV-1 Tat₁₋₈₆ (1–100 nM, clade B, #1002-2, ImmunoDX LLC, Woburn, MA), VPA (0.01–1 mM, #P4543, Sigma), SB415286 (0.01–0.05 mM, #S3567, Sigma), KN-92 (10 µM, #4130/1, Tocris Bioscience, Bristol, UK), KN-93 (10 µM, #5215/1, Tocris), MK801 (20 µM, #0924/10, Tocris) and CNQX (20 µM, #1045/1, Tocris), at 2 or 7 d after enrichment. *Cultures at 2 d after enrichment largely contain immature OLs that are O4+ and have multiple, thin processes but have not yet formed myelin-like membranes. Cultures at 7 d after enrichment largely contain more mature OLs that are also O4+, with myelin-like membranes produced from along the lengths of their processes.* Inhibitors were applied 30 min prior to Tat, for either 24 or 96 h. Concentrations of drugs were chosen as previously reported (Salter & Fern 2005, Masvekar *et al.* 2015, Waggener *et al.* 2013). For all the experiments, vehicle refers to the diluent that is used to dissolve the drug. In experiments where both DMSO and ultrapure water were used to dissolve different treatments/drugs (Fig. 5), DMSO was used as vehicle. *Responses to the Tat protein are relevant in patients receiving anti-retroviral therapy, since Tat is produced by and released from HIV-infected cells even when HIV replication is suppressed by cART* (Cysique *et al.* 2018, Johnson & Nath 2016).

Time-lapse analysis

Time-lapse analysis was performed as previously published (Zou *et al.* 2011)(Masvekar *et al.* 2015). In brief, OLs were cultured in 12-well plates and treated with Tat and GSK3β inhibitors. Plates were maintained in the environmental chamber of the Zeiss Axio Observer Z1 system (37°C, 5% CO₂; Carl Zeiss Microscopy, LLC, Thornwood, NY). For each treatment group, 50 OLs at the same stage of morphological development were chosen in sequential grids starting at the center of the plate. *OLs were initially identified in digital images based on their distinctive morphology, and immunostaining for O4 was conducted at the end of experiments to positively identify OLs or OL cellular debris. Time-lapse images of all selected cells were made at hourly intervals for 96 h using a computer-controlled stage encoder and Zeiss Axiovision 4.8 software (RRID:SCR_002677; Carl Zeiss). Cells were maintained in an environmental chamber throughout the experiment. At the experimental endpoint we assessed the pre-selected OLs for their viability in the digital images.* Cell death was determined by careful assessment of rigorous morphological criteria, including but not restricted to: (1) loss of phase brightness; (2) degenerating processes; and (3) involution or fragmentation of cell soma. *OL death was confirmed in selected experiments using a LIVE/DEAD Viability/Cytotoxicity Kit (#L3324; Life Technologies).* Data are presented as mean percentage of survival ± standard error of the mean (SEM) from at least 4 individual experiments using cultures established from different litters. *Blinding to treatment group was not possible.*

Fluorescent viability assay

Survival/death of OLs was confirmed using a LIVE/DEAD Viability/Cytotoxicity Kit (#L3324; Life Technologies). Working solutions were prepared by adding ethidium

homodimer-1 (EthD-1) and calcein-AM to sterile, tissue culture–grade D-PBS to a final concentration of 4 μ M and 2 μ M, respectively. After 96 h time-lapse imaging, 24-well plates were quickly rinsed once with sterile D-PBS and immersed in working solution for 30 min at room temperature. Images were taken using a Zeiss Axio Observer Z1 microscope with Zen 2012 software (RRID:SCR_013672; Carl Zeiss). Living or dead OLs were defined by green (calcein-AM, Em ~515 nm) or red (EthD-1, Em ~617 nm) fluorescence, respectively, and the number of green/red fluorescent pixels was assessed using ImageJ software (RRID:SCR_003070; National Institutes of Health). Each N represents a separate culture from a different litter (*biological replicates*); 20 images from a pre-determined region in the center of duplicate coverslips (*technical replicates*) were imaged, analyzed, and averaged per N.

Western blot

*Proteins from cultured cells were extracted using RIPA buffer (#R0278, Sigma) with protease and phosphatase inhibitors (#78443, Thermo Scientific). Tissues from transgenic mice were homogenized with 3 strokes (15 s/stroke) in a 2 ml ceramic bead tube (#13113–50, MO BIO Laboratories, Carlsbad, CA) using a Precellys 24 Homogenizer (#P000669-PR240-A; Bertin Technologies, Rockville, MD) before protein was extracted as per cultured cells. All protein concentrations were measured using a BCA assay (#23227, Thermo Scientific). Lysates were mixed 1:1 with 2x Laemmli buffer (#1610747 BioRad, Hercules, CA), and equal amounts of total protein (5–10 μ g) from each sample were loaded on Criterion Precast gels (#3450032, 4–20%, BioRad) and separated under constant voltage (130 V) for 1.5 h. Proteins were transferred onto polyvinylidene difluoride membranes (#1620175, BioRad) with constant current (0.6 A, 2 h, 4 °C); membranes were blocked in 0.1% casein solution (1 h, room temperature) before probing with primary antibodies. Primary antibodies specific to GSK3 β (#9315) or p-GSK3 β -S9 (#8566) (Cell Signaling Technology) and CaMKII β (RRID:AB_2275072) or p-CaMKII-T287 (ab182647) (Abcam, Cambridge, MA) were used at 1:1000 per manufacturer suggestion. Since p-CaMKII-T287 antibodies recognize all T286/7 phosphorylated CaMKII isozymes, p-CaMKII β -T287 was distinguished by its larger size. Bound antibodies were detected by appropriate IRDye secondary antibodies (RRID:AB_10956389, RRID:AB_10956589, 1:3000, LI-COR, Lincoln, NE), and imaged using an Odyssey Imager (LI-COR). Fluorescent signal intensity of targeted protein bands was analyzed using LI-COR Image Studio software. The p-GSK3 β -S9 antibody also has a weak affinity to p-GSK3 α -S21. However, p-GSK3 β -S9 (46 kDa) is easily distinguished from p-GSK3 α -S21 (51 kDa) given differences in protein size (Logie *et al.* 2017, Gupta *et al.* 2017). Western blots on brain tissue were analyzed in an investigator-blinded manner based on eartag numbers.*

Immunostaining

Immature or mature OLs cultured on glass coverslips were fixed with 4% paraformaldehyde for 10 min before being permeabilized with Triton-X 100 for 20 min. Cells were stained with the O4 monoclonal antibody (1:20) and antibodies specific against CaMKII β (#PA5–67640, 1:1000, Life Technologies), diluted in a PBS buffer containing bovine serum albumin and normal goat serum. O4 antibody, which primarily detects the sulfated galactolipid sulfatide, is grown in our lab from hybridoma cells (Knapp & Hauser 1996). Sulfatide is

expressed on both immature and mature OLs, and not found on other CNS cell types. Secondary antibodies conjugated to Alexa 594 (#A-11032) or 488 (#A11001) (1:2000, Life Technologies) were used to detect primary antibodies. Hoechst 33342 (#62249, Life Technologies) was used at 1:2000 to visualize nuclei. Coverslips were mounted onto Superfrost-Plus slides (Fisher) using Prolong Gold Antifade reagent (RRID:SCR_015961, Life Technologies). Z-stacks of fluorescent images were acquired using a Zeiss LSM 700 laser scanning confocal microscope and compiled using maximum projection.

Statistics

OL cell survival was assessed using time-lapse imaging (Figs. 1A, 5D, and 7B), for which a repeated measures two-way ANOVA with time and treatment as factors, and subsequent Bonferroni's *post-hoc* testing was used. For each treatment group, 4 individual cell cultures acquired from mice coming from different litters were used (N=4). After 96 h of time-lapse imaging, these same cells were used to perform Live/Dead assays (Fig. 1B–C), and were analyzed using Student's *t*-test. Western blot experiments comparing *in vivo* expression or activity of GSK3 β or CaMKII β in Fig. 2, or *in vitro* experiments assessing protein levels in vehicle or Tat-treated OL cultures (Figs. 3A–B, 4D, and 6A) also were analyzed using Student's *t*-test. Western blot experiments that involved multiple treatment concentrations or time points in dissociated murine OL cultures (Figs. 4A–C, 5A–C, 6B, 7A, and 7C–D) were analyzed using one-way ANOVA followed by Bonferroni's *post-hoc* testing. For all Western blot experiments comparing brain homogenates from different brain regions, three Tat– mice and three Tat+ mice, each from a different litter, were used (N=3). For Western blot experiments using OL cultures, three or four individual cultures were derived from tissue samples from different litters (N=3 or 4). The sample sizes used here are commensurate with our *a priori* power analyses indicating large differences exceeding power = 0.80 detected between Tat– and Tat+ samples on Western blots of myelin basic protein (power = 1.00) and myelin-associated glycoprotein (power = 0.89) using equivalent observations per group (Zou et al. 2015, Masvekar et al. 2015). Similarly, sample sizes for repeated measures experiments and live/dead assays (Fig. 1A-B; 7C) are based on power analyses from previous similar experiments with equal or fewer observations per group (Zou et al. 2011, Zou et al. 2015, Masvekar et al. 2015). *Dixon's two-tailed test (95% confidence level) was utilized to identify and exclude outliers from analyses.* Analyses were considered significant if $p < 0.05$. *Data were tested for equal variance and homoscedasticity using Bartlett's test and the Brown-Forsythe test. Normal distribution was verified via the Shapiro-Wilk test with visual assessment of Q-Q plots.*

RESULTS:

Effects of Tat on OL survival.

We first confirmed that HIV-1 Tat caused OL degeneration using morphologic criteria in phase contrast digital photomicrographs, and assessed the time course of cell losses. As noted previously, loss of immature OLs in untreated cultures exceeds that of mature OLs over the 3 day experimental period (Zou et al. 2015). The survival of mature OLs (7 d in culture, O4⁺/MBP⁺) was unaffected by Tat at 96 h, while the viability of immature OLs (2 d in culture, O4⁺/MBP⁻) was significantly reduced by Tat in a concentration-dependent

manner (Fig. 1A). The effects of 100 nM Tat on immature OL survival reached statistical significance as early as 24 h post-treatment, quite a bit earlier than observed with 10 nM Tat (~60 h, Fig. 1A). Live/dead assays were performed on cells after these experiments (at 96 h) to verify that the degenerating OLs identified by phase contrast microscopy had lost cell membrane integrity, confirming cell death. The Tat-treated (100 nM) group exhibited significantly more red fluorescent signal (EthD-1⁺, dead), and less green fluorescent signal (Calcein-AM⁺, live), when compared to control groups (Figs. 1B, C).

Tat expression *in vivo* activates GSK3 β and CaMKII β in multiple brain regions.

Since expression of Tat *in vivo* leads to increased caspase-3 activity in O4+ oligodendroglial lineage cells (Hauser *et al.* 2009), we investigated Tat effects on GSK3 β or CaMKII β signaling by Western blot using a DOX-inducible Tat-transgenic mouse model. We examined white matter-containing regions where morphological and functional changes are prevalent in both animal models of HIV and in HIV-infected individuals (cortex, striatum, hippocampus) and compared them to the cerebellum, a region that is less affected. A significantly decreased ratio of pGSK3 β -S9 to total GSK3 β (p-GSK3 β /t-GSK3 β) was detected in cortex, striatum, and hippocampus, suggesting that GSK3 β activity was upregulated in these areas by Tat (Fig. 2A,B). GSK3 β activity in cerebellum showed an opposing trend towards a decrease (Fig. 2B, $p = 0.077$). CaMKII β protein levels were significantly increased in three of the four brain regions examined in Tat+ mice (Fig. 2C, cerebellum: $p = 0.09$). Further, total CaMKII β activity, as assessed by phosphorylation at CaMKII β 's T287 site, was increased in all 4 regions (cortex, cerebellum, striatum, and hippocampus) in Tat+ mice (Fig. 2D). Since other cells types express both GSK3 β and CaMKII β , these changes may not be unique to OLs.

Expression and localization of CaMKII β varies with developmental stage

OLs at different developmental stages show distinct patterns of expression and subcellular distribution of iGluRs, GSK3 β and CaMKII β (Zhang *et al.* 2014, Karadottir *et al.* 2005, De Biase *et al.* 2010). We thus assessed the level of GSK3 β and CaMKII β proteins using Western blotting and CaMKII β localization by immunocytochemistry. Total GSK3 β levels in immature OLs were significantly higher than in mature OLs, independent of Tat treatment (Fig. 3A). In contrast, total CaMKII β levels were significantly higher in mature OLs compared to immature OLs, also independent of Tat treatment (Fig. 3B). In immature OLs, CaMKII β localized mainly to cellular processes, while in mature OLs CaMKII β was found in both the cytoplasm of larger processes and the cell body (Fig. 3C). A varying ratio of GSK3 β to CaMKII β , and distinct subcellular distribution of CaMKII β may contribute to the differential vulnerability of immature and mature OLs to Tat. The activation of GSK3 β versus CaMKII β as a function of OL development is explored in experiments described below.

Tat upregulates GSK3 β activity in immature, but not mature OLs.

It is well-established that GSK3 β -S9-phosphorylation reduces GSK3 β activity (Doble & Woodgett 2003, Hur & Zhou 2010, Masvekar *et al.* 2015). Thus, we and others have used the ratio of p-GSK3 β -S9:t-GSK3 β to indicate GSK3 β activity (decreased ratio indicating increased GSK3 β activity). The p-GSK3 β -S9:t-GSK3 β ratio was significantly decreased in

immature OLs treated with 10 or 100 nM Tat as compared with vehicle or 1 nM Tat-treated groups (Fig. 4A), indicating upregulated GSK3 β activity. Total GSK3 β was not affected by Tat treatment over 96 h (Fig. 4D). The upregulation of GSK3 β activity by Tat (100 nM) was detected as early as 2 h, and persisted over 96 h (Fig. 4C). In comparison, Tat did not affect GSK3 β activity in mature OL cultures (Fig. 4B).

Inhibiting GSK3 β activation rescues immature OLs from Tat-induced death *in vitro*.

We previously reported that the immature OL death induced by Tat could be reversed by the *N*-methyl-D-aspartate receptor (NMDA-R) antagonist, MK801, or the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/kainate receptor (AMPA/KA-R) antagonist, CNQX (Zou et al. 2015). Since iGluR activation has also been shown to regulate GSK3 β activity (De Montigny *et al.* 2013), we assessed the effects of MK801/CNQX on GSK3 β in Tat-treated OLs. Western blots showed that the up-regulated GSK3 β activity caused by Tat was completely abolished when MK801 or CNQX were added concurrently (Fig. 5A), implicating GSK3 β in mediating the toxic effect of Tat on immature OLs.

We next investigated whether blocking GSK3 β could reverse the effect of Tat on immature OL survival, using two drugs that block GSK3 β activity via different mechanisms. VPA is used widely as a GSK3 β inhibitor, working through activation of its upstream WNT signaling pathway (Hall *et al.* 2002), but has numerous other actions including HDAC, nitric oxide synthase, and aromatase inhibition. SB415286 is a more specific small molecule inhibitor that competitively binds to an ATP binding domain and adjacent sites, inhibiting activities downstream of GSK3 β activation (Liang & Chuang 2007, Coghlan *et al.* 2000). Both VPA (0.1–1 mM) and SB415286 (0.01–0.05 mM) effectively blocked Tat induction of GSK3 β activity in immature OLs after 24 h treatment (Fig. 5B and C). Continuous VPA (100 μ M) and SB415286 (10 μ M) treatment reduced the GSK3 β activity (Fig. 5C) and reversed OL death induced by 96 h Tat (Fig. 5D).

Tat activates CaMKII β *in vitro* via iGluRs in both immature and mature OLs.

Given that Tat activates iGluRs in both immature and mature OLs, it was intriguing that Tat did not increase GSK3 β activity in mature OLs. Activated CaMKII has been reported to phosphorylate GSK3 β -S9 and thereby inhibit GSK3 β , and to promote neuronal survival (Song *et al.*, 2010). Our previous studies have also shown that Tat activates OL iGluRs and up-regulates CaMKII β activity (Zou et al. 2015). Since CaMKII β is the predominant CaMKII isoform in OLs, based on the neuronal results, we hypothesized that Tat-induced CaMKII β activation may maintain higher levels of GSK3 β phosphorylation (reduced GSK3 β activity) in mature OLs, thus making them less vulnerable to Tat. To test this hypothesis, we first verified the effects of Tat on CaMKII β activities in OLs. Western blots showed that CaMKII β activity was significantly increased in both immature and mature OLs treated with Tat (100 nM, 1h), as demonstrated by an increase in the p-CaMKII β -T287/t-CaMKII β ratio (Hudmon & Schulman 2002b) (Fig. 6A). The Tat-induced CaMKII β activation in OLs was mediated through iGluRs activation, since MK801 completely reversed, and CNQX partially blocked, the upregulated CaMKII β activity in both immature and mature OL treated with 100 nM Tat (Fig. 6B).

CaMKII β suppresses Tat-induced GSK3 β activity in mature OLs

To test the effects of inhibiting CaMKII β on Tat-induced GSK3 β activation, we added the CaMKII β inhibitor KN93 and its inactive derivative KN92 concurrent with Tat. Although Tat by itself did not affect GSK3 β activity in mature OLs (Fig. 7A), KN93 + Tat significantly increased GSK3 β activity at 2 h and 6 h. This suggests that normal levels of CaMKII β activity were sufficient to suppress Tat-induced GSK3 β activation in mature OLs. In contrast, Tat alone increased GSK3 β activity in immature OLs, and this was further enhanced by KN93 (Fig. 7A). KN92 had no effect on GSK3 β activities at either stage of maturity.

CaMKII β -deficient OLs have enhanced vulnerability to Tat

Longer-term applications of KN93 were toxic to OLs *in vitro*, so we used primary OLs cultured from *Camk2b^{fl/fl}* mice to test the hypothesis that knocking down CaMKII β would make mature OLs vulnerable to Tat. In cell culture, loxP-flanked genes can be excised by adding cell-permeable Cre recombinase to the media (O'Meara *et al.* 2013). RT-PCR showed that 50 nM Cre significantly decreased the mRNA level of *Camk2b* in mature OL cultures at 24 h (Fig. 7B, inset). Importantly, the viability of mature OLs exposed to Tat was significantly decreased between 24 and 96 h when *Camk2b* was knocked down (Fig. 7B). Knockdown of *Camk2b* by itself decreased mature OL viability after 72 h, although always to a lesser extent than with co-exposure to Tat. To further clarify that these effects on viability were mediated by knockdown of CaMKII β , Western blot and live/dead assays were performed at 0–96 h on mature, wild-type, or *Camk2b^{fl/fl}* OLs treated with Cre. Adding Cre to the media significantly decreased CaMKII β protein levels (48 – 96 h) and cell viability (72 – 96 h) in *Camk2b^{fl/fl}*, but not wild-type, mature OLs (Fig. 7C-D).

DISCUSSION

Myelin disruptions are a common finding in the CNS of HIV-infected individuals, and indications of OL dysfunction have been noted both in post-mortem tissue and experimentally. While blood-brain barrier leakage and edema probably contribute to these outcomes, we have also shown that OLs are directly injured by exposure to HIV-1 Tat, which increases $[Ca^{2+}]_i$ levels coincident with increased CaMKII β activity in both immature OLs, and *mature OLs that are producing myelin-like membranes* (Zou et al. 2015). Viability of immature OLs was reduced by Tat and was rescued by attenuating $[Ca^{2+}]_i$ increases. However, mature OLs survived very high $[Ca^{2+}]_i$ increases and instead reduced their production of myelin-like membranes (Zou et al. 2015). Here we have explored the basis for these quite different responses of *immature* versus mature OLs to Tat-induced $[Ca^{2+}]_i$ increases.

The finding that $[Ca^{2+}]_i$ was increased and CaMKII β activated at both developmental stages suggested that different pathways downstream of CaMKII β might be activated in OLs as they matured. GSK3 β activity is upregulated by iGluR-mediated $[Ca^{2+}]_i$ increases (Hartigan & Johnson 1999), and negatively regulates OL proliferation and myelination (Azim & Butt 2011). It also has been invoked as a cause of Tat-induced neurotoxicity in numerous studies (Masvekar et al. 2015, Chao *et al.* 2014). Since CaMKII activation can inhibit GSK3 β , we

hypothesized that the relative levels of total and activated CaMKII versus GSK3 β might be altered to favor cell survival in more mature OLs.

As GSK3 β is ubiquitously expressed among CNS cell types, we used highly purified cultures to assess whether Tat regulates GSK3 β activity in OLs. Tat caused concentration-dependent GSK3 β activation in immature, but not mature OLs, in parallel with decreased cell viability (Fig. 1A, 4A-C). MK801 or CNQX, which attenuated the Tat-induced $[Ca^{2+}]_i$ increase and rescued immature OLs from Tat-induced death (Zou et al. 2015), also reversed GSK3 β activation by Tat (Fig. 5A). This finding is consistent with the idea that GSK3 β activation can be caused by iGluR-mediated $[Ca^{2+}]_i$ increases (Hartigan & Johnson 1999). Further, VPA and SB415286, two GSK3 β inhibitors that block GSK3 β activity via different mechanisms, both reversed Tat-induced immature OL death (Fig. 5D). Together, these data strongly support a Tat-induced signaling mechanism whereby interactions between Tat and iGluRs lead to $[Ca^{2+}]_i$ increase, GSK3 β activation in immature OLs, and subsequent cell death. Theoretically, VPA and SB415286 also inhibit GSK3 α (Coghlan et al. 2000). However, Tat did not alter GSK3 α activity in OLs (data not shown), suggesting that GSK3 β is the principal target.

Since Tat elevates $[Ca^{2+}]_i$ similarly in mature and immature OLs (Zou et al. 2015), it was intriguing that Tat did not activate GSK3 β in mature OLs. Based on our previous finding that Tat activates CaMKII β (Zou et al. 2015), the predominant isoform of CaMKII in OLs (Waggener et al. 2013), and that CaMKII β has been reported to inhibit GSK3 β (Song et al. 2010), we hypothesized that $[Ca^{2+}]_i$ -mediated GSK3 β activation in OLs might be determined by the balance between CaMKII β and GSK3 β signaling. The relatively higher levels of CaMKII β versus GSK3 β measured in mature OLs may contribute to greater inhibition of GSK3 β activity and less vulnerability to the toxic effects of Tat. Independent of kinase activities, CaMKII β also can function structurally by stabilizing the actin cytoskeleton (Hudmon & Schulman 2002a), which has been shown to influence OL maturation and myelination (Waggener et al. 2013). Persistent cytoskeletal disruption may thus contribute to mature OL loss after 72 h of *Camk2b* knockdown. This might be further clarified by experiments using the *Camk2b*^{A303R} mice, in which the mutated CaMKII β (*CaMKII β* ^{A303R}) results in lost kinase catalytic activity, with preservation of actin-binding capacity (Lin & Redmond 2008, O'Leary et al. 2006).

NMDA-R stimulation has been reported to influence both GSK3 β and CaMKII activity, inhibiting GSK3 β (De Montigny et al. 2013) and persistently activating CaMKII, due to stable interactions with the NR2B subunit (Bayer et al. 2006, Lee et al. 2009). Since mature OLs express higher levels of NMDA-Rs than immature OLs (De Biase et al. 2010, Micu et al. 2006, Salter & Fern 2005), the Tat-induced NMDA-R activation that we have previously shown in OLs (Zou et al. 2015), and that others have observed in neurons (Haughey et al. 2001, Kim et al. 2008), might also contribute to downregulated GSK3 β activity in mature OLs.

In vivo expression of HIV-1 Tat expression altered overall CaMKII β and GSK3 β activities in multiple brain regions. Although CaMKII β activity was consistently elevated by Tat (Fig. 2D), it did not suppress overall GSK3 β activity, except perhaps in cerebellum (Fig. 2B). *In*

in vivo measures reflect the combined responses of multiple cell types in the brain that express both enzymes, not all of which will respond equally to Tat. In addition, GSK3 β expression is quite high compared to CaMKII β expression in many CNS cells (Zhang et al. 2014), suggesting that CaMKII β suppression of GSK3 β activation may be unique to OLs. The *in vitro* studies likely provide greater insight into OL-specific responses.

Dysregulation of GSK3 β activity has been reported to be involved in multiple neurodegenerative processes, including HAND (Jacobs *et al.* 2012, Crews *et al.* 2009, Schifitto *et al.* 2006). Among the regions we examined, GSK3 β activity was upregulated in all except the cerebellum (Fig. 2B). This observation is intriguing for several reasons. Firstly, clinical observations of HIV-related neuropathology are rarely reported in cerebellum, but frequently reported in the other regions examined. Secondly, CaMKII β is the predominant isoform of CaMKII in both OLs and cerebellar granule neurons (Waggener et al. 2013, Burgin et al. 1990) and our experiments reveal CaMKII β activity to be significantly upregulated by Tat expression in cerebellum (Fig. 2D). We have also previously shown that cerebellar astrocytes secrete significantly less cytokines/chemokines than astrocytes from cortex or spinal cord when exposed to Tat *in vitro* (Fitting *et al.* 2010). Together, these findings suggest that the more limited glial inflammatory response and high levels of CaMKII β in cerebellar neurons may protect that region from GSK3 β -induced damage due to Tat or other HIV proteins.

Data reported here suggest that the relative levels of CaMKII β and GSK3 β may be critical in determining OL vulnerability to Tat (summarized in Fig. 8). Exposure to Tat leads to Ca²⁺ influx into the cytoplasm, which activates both GSK3 β (p-GSK3 β -S9 dephosphorylation) and CaMKII β (CaMKII β -T287 phosphorylation). In mature OLs, a large amount of activated cytoplasmic CaMKII β inhibits the activated GSK3 β , which protects cells from death. In immature OLs, the amount of activated CaMKII β is not sufficient to inhibit GSK3 β , and consequently, downstream cell death signaling pathways are triggered.

Our results with HIV-1 Tat are in accord with the finding that conditional depletion of GSK3 β protected myelinating OLs from caspase-dependent death in the cuprizone demyelination model (Xing *et al.* 2018). Although CaMKII β levels or activity were not explored in the cuprizone study, it is striking that GSK3 β activation is implicated in OL death resulting from these disparate insults. Survival during development is closely linked to differentiation, and GSK3 β activation has been observed to positively (Zhou *et al.* 2014) or negatively (Azim & Butt 2011, Azim *et al.* 2014) affect OL maturation, in part depending on the dynamics of Wnt/ β -catenin signaling. GSK3 β activation appears to be at the crossroads of multiple developmental events whose outcomes may depend upon multiple factors related to specific stages in the OL lineage.

White matter injuries, such as decreased volume (Sarma *et al.* 2014), increased occurrence of abnormal myelinated tracts (Gongvatana *et al.* 2009), and degenerated OLs (Gyorkey et al. 1987, Jayadev et al. 2007), contribute to HAND pathogenesis and can occur during early, asymptomatic stages of HIV infection. cART itself has also been recently shown to impact myelin integrity and maintenance (Jensen *et al.* 2015), suggesting that these injuries may be extremely difficult to prevent in HIV-infected patients. Thus, the ability of immature OLs to

survive, differentiate and remyelinate may be an important factor in limiting CNS damage due to HIV exposure. Our findings suggest that immature OLs are vulnerable to Tat-induced death because CaMKII β levels are insufficient to restrict GSK3 β activation. This vulnerability at an early developmental stage may compromise myelin repair efforts. Therapeutic strategies that directly target GSK3 β activity in OLs may be difficult to design since GSK3 β is ubiquitous. However, an indirect approach by modulating NMDA-R and/or increasing CaMKII β *to overcome any deleterious effects of Tat-induced GSK3 β activation* might provide important adjunctive therapy to HIV-infected individuals afflicted with HAND.

Acknowledgements:

Funding support from the NIH: F31 NS084838 (SZ), R01 DA024461 (PEK), R01 DA044939 (KFH and PEK), R21 NS084335 (BF), and K02 DA027374 (KFH) are greatly appreciated. Electron microscopy was performed at the VCU-Dept. Anatomy and Neurobiology Microscopy Facility, supported in part with funding from NIH-NINDS Center core grant 5P30NS047463.

Abbreviations

ANOVA	Analysis of variance
cART	Combined antiretroviral therapy
[Ca²⁺]_I	Intracellular calcium concentration
CaMKIIβ	Ca ²⁺ /calmodulin-dependent kinase II β
<i>Camk2b</i>^{fl/fl}	Transgenic mouse with a loxP-modified <i>Camk2b</i> allele
CNQX	Cyanquixilene
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DOX	Doxycycline
EthD-1	Ethidium homodimer-1
GSK3β	Glycogen synthase kinase 3 β
GSK3α	Glycogen synthase kinase 3 α
HAND	HIV-associated neurocognitive disorders
HIV	Human immunodeficiency virus
IGluR	ionotropic glutamate receptor
NAC	N-acetyl cysteine

NMDA-R	N-methyl-D-aspartate receptor
OL	Oligodendrocyte
p-CaMKII-T287	CaMKII phosphorylated at Threonine 287
p-GSK3β-S9	GSK3 β phosphorylated at Serine 9
RIPA	Radioimmunoprecipitation assay
RRID	<i>Research Resource Identifier</i>
Tat	Transactivator of transcription
VPA	Valproic acid

References:

- Albright AV, Strizki J, Harouse JM, Lavi E, O'Connor M and Gonzalez-Scarano F (1996) HIV-1 infection of cultured human adult oligodendrocytes. *Virology*, 217, 211–219. [PubMed: 8599205]
- Azim K and Butt AM (2011) GSK3beta negatively regulates oligodendrocyte differentiation and myelination in vivo. *Glia*
- Azim K, Rivera A, Raineteau O and Butt AM (2014) GSK3beta regulates oligodendrogenesis in the dorsal microdomain of the subventricular zone via Wnt-beta-catenin signaling. *Glia*, 62, 778–779. [PubMed: 24677550]
- Bagashev A and Sawaya BE (2013) Roles and functions of HIV-1 Tat protein in the CNS: an overview. *Virology*, 10, 358. [PubMed: 24359561]
- Bayer KU, LeBel E, McDonald GL, O'Leary H, Schulman H and De Koninck P (2006) Transition from reversible to persistent binding of CaMKII to postsynaptic sites and NR2B. *J Neurosci*, 26, 1164–1174. [PubMed: 16436603]
- Burgin KE, Waxham MN, Rickling S, Westgate SA, Mobley WC and Kelly PT (1990) In situ hybridization histochemistry of Ca²⁺/calmodulin-dependent protein kinase in developing rat brain. *J Neurosci*, 10, 1788–1798. [PubMed: 2162385]
- Chao J, Yang L, Yao H and Buch S (2014) Platelet-derived growth factor-BB restores HIV Tat - mediated impairment of neurogenesis: role of GSK-3beta/beta-catenin. *Journal of Neuroimmune Pharmacol*, 9, 259–268.
- Chopard C, Tong PBV, Toth P et al. (2018) Cyclophilin A enables specific HIV-1 Tat palmitoylation and accumulation in uninfected cells. *Nat Commun*, 9, 2251. [PubMed: 29884859]
- Coghlan MP, Culbert AA, Cross DA et al. (2000) Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol*, 7, 793–803. [PubMed: 11033082]
- Crews L, Patrick C, Achim CL, Everall IP and Masliah E (2009) Molecular pathology of neuro-AIDS (CNS-HIV). *Int J Mol Sci*, 10, 1045–1063. [PubMed: 19399237]
- Cysique LA, Juge L, Lennon MJ et al. (2018) HIV brain latency as measured by CSF BcL11b relates to disrupted brain cellular energy in virally suppressed HIV infection. *AIDS*
- Dayton AI, Sodroski JG, Rosen CA, Goh WC and Haseltine WA (1986) The trans-activator gene of the human T cell lymphotropic virus type III is required for replication. *Cell*, 44, 941–947. [PubMed: 2420471]
- De Biase LM, Nishiyama A and Bergles DE (2010) Excitability and synaptic communication within the oligodendrocyte lineage. *J Neurosci*, 30, 3600–3611. [PubMed: 20219994]
- De Montigny A, Elhiri I, Allyson J, Cyr M and Massicotte G (2013) NMDA reduces Tau phosphorylation in rat hippocampal slices by targeting NR2A receptors, GSK3beta, and PKC activities. *Neural Plast*, 2013, 261593. [PubMed: 24349798]

- Doble BW and Woodgett JR (2003) GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci*, 116, 1175–1186. [PubMed: 12615961]
- Ensofi B, Buonaguro L, Barillari G, Fiorelli V, Gendelman R, Morgan RA, Wingfield P and Gallo RC (1993) Release, uptake, and effects of extracellular human immunodeficiency virus type 1 Tat protein on cell growth and viral transactivation. *J Virol*, 67, 277–287. [PubMed: 8416373]
- Fisher AG, Feinberg MB, Josephs SF et al. (1986) The trans-activator gene of HTLV-III is essential for virus replication. *Nature*, 320, 367–371. [PubMed: 3007995]
- Fitting S, Zou S, Chen W, Vo P, Hauser KF and Knapp PE (2010) Regional heterogeneity and diversity in cytokine and chemokine production by astroglia: differential responses to HIV-1 Tat, gp120, and morphine revealed by multiplex analysis. *J Proteome Res*, 9, 1795–1804. [PubMed: 20121167]
- Frankel AD and Pabo CO (1988) Cellular uptake of the tat protein from human immunodeficiency virus. *Cell*, 55, 1189–1193. [PubMed: 2849510]
- Gongvatana A, Schweinsburg BC, Taylor MJ et al. (2009) White matter tract injury and cognitive impairment in human immunodeficiency virus-infected individuals. *J Neurovirol*, 15, 187–195. [PubMed: 19306228]
- Gorry PR, Ong C, Thorpe J, Bannwarth S, Thompson KA, Gatignol A, Vesselingh SL and Purcell DF (2003) Astrocyte infection by HIV-1: mechanisms of restricted virus replication, and role in the pathogenesis of HIV-1-associated dementia. *Curr HIV Res*, 1, 463–473. [PubMed: 15049431]
- Gupta A, Anjomani-Virmouni S, Koundouros N and Pouligiannis G (2017) PARK2 loss promotes cancer progression via redox-mediated inactivation of PTEN. *Mol Cell Oncol*, 4, e1329692. [PubMed: 29209642]
- Gyorkey F, Melnick JL and Gyorkey P (1987) Human immunodeficiency virus in brain biopsies of patients with AIDS and progressive encephalopathy. *J Infect Dis*, 155, 870–876. [PubMed: 3644852]
- Hall AC, Brennan A, Goold RG, Cleverley K, Lucas FR, Gordon-Weeks PR and Salinas PC (2002) Valproate regulates GSK-3-mediated axonal remodeling and synapsin I clustering in developing neurons. *Mol Cell Neurosci*, 20, 257–270. [PubMed: 12093158]
- Hartigan JA and Johnson GV (1999) Transient increases in intracellular calcium result in prolonged site-selective increases in Tau phosphorylation through a glycogen synthase kinase 3beta-dependent pathway. *J Biol Chem*, 274, 21395–21401. [PubMed: 10409701]
- Haughey NJ, Nath A, Mattson MP, Slevin JT and Geiger JD (2001) HIV-1 Tat through phosphorylation of NMDA receptors potentiates glutamate excitotoxicity. *J Neurochem*, 78, 457–467. [PubMed: 11483648]
- Hauser KF, Hahn YK, Adjan VV, Zou S, Buch SK, Nath A, Bruce-Keller AJ and Knapp PE (2009) HIV-1 Tat and morphine have interactive effects on oligodendrocyte survival and morphology. *Glia*, 57, 194–206. [PubMed: 18756534]
- Heaton RK, Clifford DB, Franklin DR, Jr. et al. (2010) HIV-associated neurocognitive disorders persist in the era of potent antiretroviral therapy: CHARTER Study. *Neurology*, 75, 2087–2096. [PubMed: 21135382]
- Hudmon A and Schulman H (2002a) Neuronal CA2+/calmodulin-dependent protein kinase II: the role of structure and autoregulation in cellular function. *Annu Rev Biochem*, 71, 473–510. [PubMed: 12045104]
- Hudmon A and Schulman H (2002b) Structure-function of the multifunctional Ca2+/calmodulin-dependent protein kinase II. *Biochem J*, 364, 593–611. [PubMed: 11931644]
- Hur EM and Zhou FQ (2010) GSK3 signalling in neural development. *Nature Rev Neurosci*, 11, 539–551. [PubMed: 20648061]
- Jacobs KM, Bhave SR, Ferraro DJ, Jaboin JJ, Hallahan DE and Thotala D (2012) GSK-3beta: A Bifunctional Role in Cell Death Pathways. *Int J Cell Biol*, 2012, 930710. [PubMed: 22675363]
- Jayadev S, Yun B, Nguyen H, Yokoo H, Morrison RS and Garden GA (2007) The glial response to CNS HIV infection includes p53 activation and increased expression of p53 target genes. *J Neuroimmune Pharmacol*, 2, 359–370. [PubMed: 18040854]
- Jensen BK, Monnerie H, Mannell MV et al. (2015) Altered Oligodendrocyte Maturation and Myelin Maintenance: The Role of Antiretrovirals in HIV-Associated Neurocognitive Disorders. *J Neuropathol Exp Neurol*, 74, 1093–1118. [PubMed: 26469251]

- Johnson TP and Nath A (2016) Protocol for Detection of HIV-Tat Protein in Cerebrospinal Fluid by a Sandwich Enzyme-Linked Immunosorbent Assay. *Methods Mol Biol*, 1354, 343–352. [PubMed: 26714723]
- Karadottir R, Cavalier P, Bergersen LH and Attwell D (2005) NMDA receptors are expressed in oligodendrocytes and activated in ischaemia. *Nature*, 438, 1162–1166. [PubMed: 16372011]
- Kim HJ, Martemyanov KA and Thayer SA (2008) Human immunodeficiency virus protein Tat induces synapse loss via a reversible process that is distinct from cell death. *J Neurosci*, 28, 12604–12613. [PubMed: 19036954]
- Knapp PE and Hauser KF (1996) mu-Opioid receptor activation enhances DNA synthesis in immature oligodendrocytes. *Brain Res*, 743, 341–345. [PubMed: 9017266]
- Lee SJ, Escobedo-Lozoya Y, Szatmari EM and Yasuda R (2009) Activation of CaMKII in single dendritic spines during long-term potentiation. *Nature*, 458, 299–304. [PubMed: 19295602]
- Liang MH and Chuang DM (2007) Regulation and function of glycogen synthase kinase-3 isoforms in neuronal survival. *The Journal of biological chemistry*, 282, 3904–3917. [PubMed: 17148450]
- Lin YC and Redmond L (2008) CaMKIIbeta binding to stable F-actin in vivo regulates F-actin filament stability. *Proc Natl Acad Sci USA*, 105, 15791–15796. [PubMed: 18840684]
- Logie L, Van Aalten L, Knebel A et al. (2017) Rab-GTPase binding effector protein 2 (RABEP2) is a primed substrate for Glycogen Synthase kinase-3 (GSK3). *Sci Rep*, 7, 17682. [PubMed: 29247183]
- Masvekar RR, El-Hage N, Hauser KF and Knapp PE (2015) GSK3beta-activation is a point of convergence for HIV-1 and opiate-mediated interactive neurotoxicity. *Mol Cell Neurosci*, 65, 11–20. [PubMed: 25616162]
- McArthur JC, Steiner J, Sacktor N and Nath A (2010) Human immunodeficiency virus-associated neurocognitive disorders: Mind the gap. *Ann Neurol*, 67, 699–714. [PubMed: 20517932]
- Micu I, Jiang Q, Coderre E et al. (2006) NMDA receptors mediate calcium accumulation in myelin during chemical ischaemia. *Nature*, 439, 988–992. [PubMed: 16372019]
- O’Leary H, Lasda E and Bayer KU (2006) CaMKIIbeta association with the actin cytoskeleton is regulated by alternative splicing. *Molec Biol Cell*, 17, 4656–4665. [PubMed: 16928958]
- O’Meara RW, Michalski JP, Anderson C, Bhanot K, Rippstein P and Kothary R (2013) Integrin-linked kinase regulates process extension in oligodendrocytes via control of actin cytoskeletal dynamics. *J Neurosci*, 33, 9781–9793. [PubMed: 23739974]
- Sacktor N, Lyles RH, Skolasky R et al. (2001) HIV-associated neurologic disease incidence changes: Multicenter AIDS Cohort Study, 1990–1998. *Neurology*, 56, 257–260. [PubMed: 11160967]
- Sacktor N, Miyahara S, Deng L et al. (2011) Minocycline treatment for HIV-associated cognitive impairment: results from a randomized trial. *Neurology*, 77, 1135–1142. [PubMed: 21900636]
- Salter MG and Fern R (2005) NMDA receptors are expressed in developing oligodendrocyte processes and mediate injury. *Nature*, 438, 1167–1171. [PubMed: 16372012]
- Sarma MK, Nagarajan R, Keller MA, Kumar R, Nielsen-Saines K, Michalik DE, Deville J, Church JA and Thomas MA (2014) Regional brain gray and white matter changes in perinatally HIV-infected adolescents. *Neuroimage Clin*, 4, 29–34. [PubMed: 24380059]
- Sattentau QJ, Dalgleish AG, Weiss RA and Beverley PC (1986) Epitopes of the CD4 antigen and HIV infection. *Science*, 234, 1120–1123. [PubMed: 2430333]
- Schifitto G, Peterson DR, Zhong J, Ni H, Cruttenden K, Gaugh M, Gendelman HE, Boska M and Gelbard H (2006) Valproic acid adjunctive therapy for HIV-associated cognitive impairment: a first report. *Neurology*, 66, 919–921. [PubMed: 16510768]
- Song B, Lai B, Zheng Z, Zhang Y, Luo J, Wang C, Chen Y, Woodgett JR and Li M (2010) Inhibitory phosphorylation of GSK-3 by CaMKII couples depolarization to neuronal survival. *J Biol Chem*, 285, 41122–41134. [PubMed: 20841359]
- Tan IL and McArthur JC (2012) HIV-associated neurological disorders: a guide to pharmacotherapy. *CNS Drugs*, 26, 123–134. [PubMed: 22201342]
- Waggener CT, Dupree JL, Elgersma Y and Fuss B (2013) CaMKIIbeta Regulates Oligodendrocyte Maturation and CNS Myelination. *J Neurosci*, 33, 10453–10458. [PubMed: 23785157]

- Xing B, Brink LE, Maers K, Sullivan ML, Bodnar RJ, Stolz DB and Cambi F (2018) Conditional depletion of GSK3b protects oligodendrocytes from apoptosis and lessens demyelination in the acute cuprizone model. *Glia*
- Yilmaz A, Svennerholm B, Hagberg L and Gisslen M (2006) Cerebrospinal fluid viral loads reach less than 2 copies/ml in HIV-1-infected patients with effective antiretroviral therapy. *Antivir Ther*, 11, 833–837. [PubMed: 17302245]
- Zhang Y, Chen K, Sloan SA et al. (2014) An RNA-sequencing transcriptome and splicing database of glia, neurons, and vascular cells of the cerebral cortex. *J Neurosci*, 34, 11929–11947. [PubMed: 25186741]
- Zhou L, Shao CY, Xu SM et al. (2014) GSK3beta promotes the differentiation of oligodendrocyte precursor cells via beta-catenin-mediated transcriptional regulation. *Mol Neurobiol*, 50, 507–519. [PubMed: 24691545]
- Zou S, Fitting S, Hahn YK, Welch SP, El-Hage N, Hauser KF and Knapp PE (2011) Morphine potentiates neurodegenerative effects of HIV-1 Tat through actions at mu-opioid receptor-expressing glia. *Brain*, 134, 3613–3628.
- Zou S, Fuss B, Fitting S, Hahn YK, Hauser KF and Knapp PE (2015) Oligodendrocytes Are Targets of HIV-1 Tat: NMDA and AMPA Receptor-Mediated Effects on Survival and Development. *J Neurosci*, 35, 11384–11398. [PubMed: 26269645]

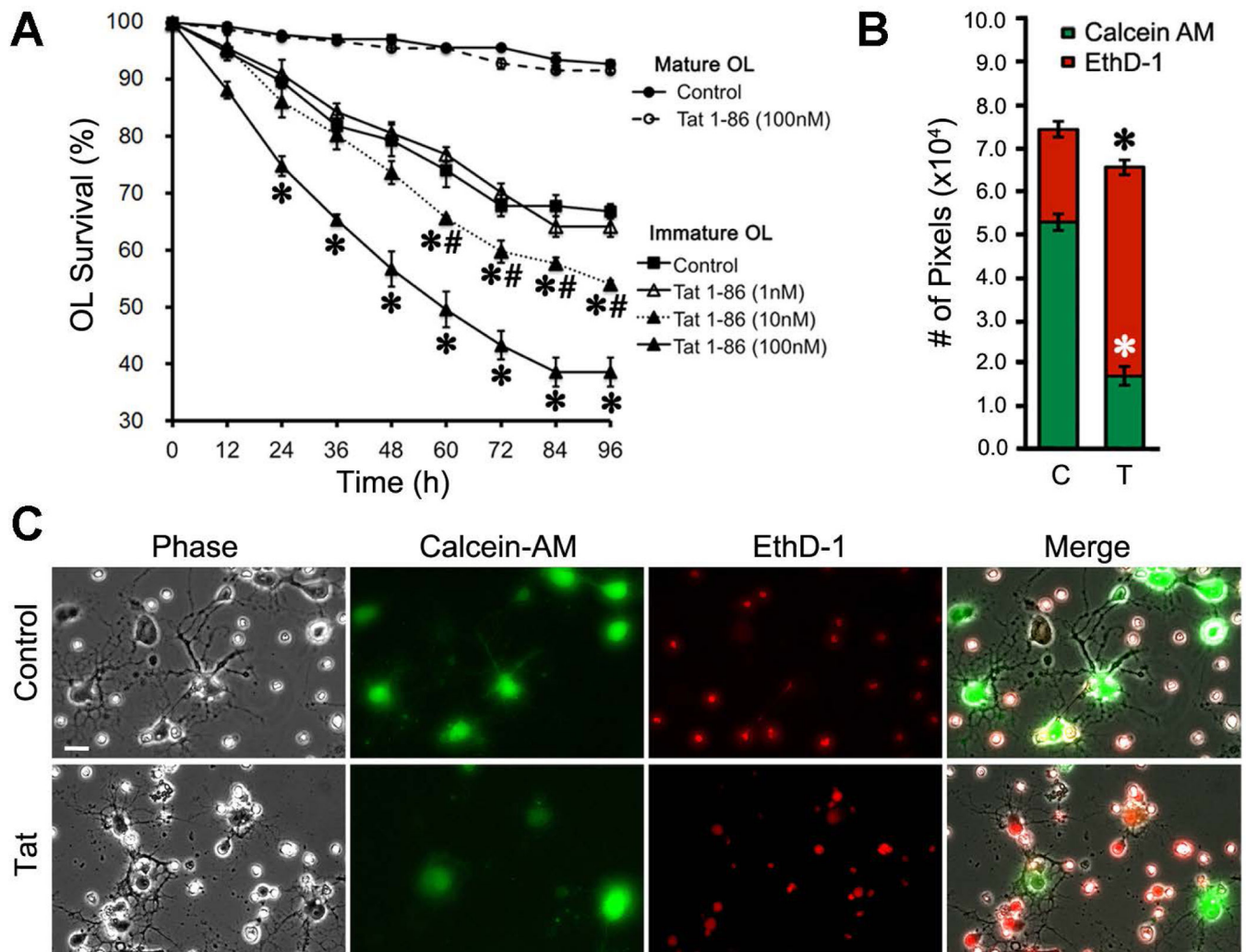


Figure 1. Effects of Tat on OL survival.

(A) Tat treatment led to a dose-dependent decrease of immature OL survival, but did not affect survival of mature OLs. In 4 independent experiments, 50 OLs were tracked for every treatment during the course of the entire 96 h time period; survival effects were analyzed by repeated measures two-way ANOVA. (B-C) Some results from (A) were confirmed by a fluorescent viability assay using Calcein-AM (green) and EthD-1 (red), and quantified with ImageJ software. Sample images in (C) were chosen from vehicle and Tat-treated immature OL groups assayed at 96 h. (* $p < 0.05$ vs. corresponding control group; # $p < 0.05$ vs. corresponding 100 nM Tat group; Scale bar: 10 μm ; C: control; T: 100 nM Tat). For each experiment, $N=4$ independent cell cultures prepared from mice of different litters.

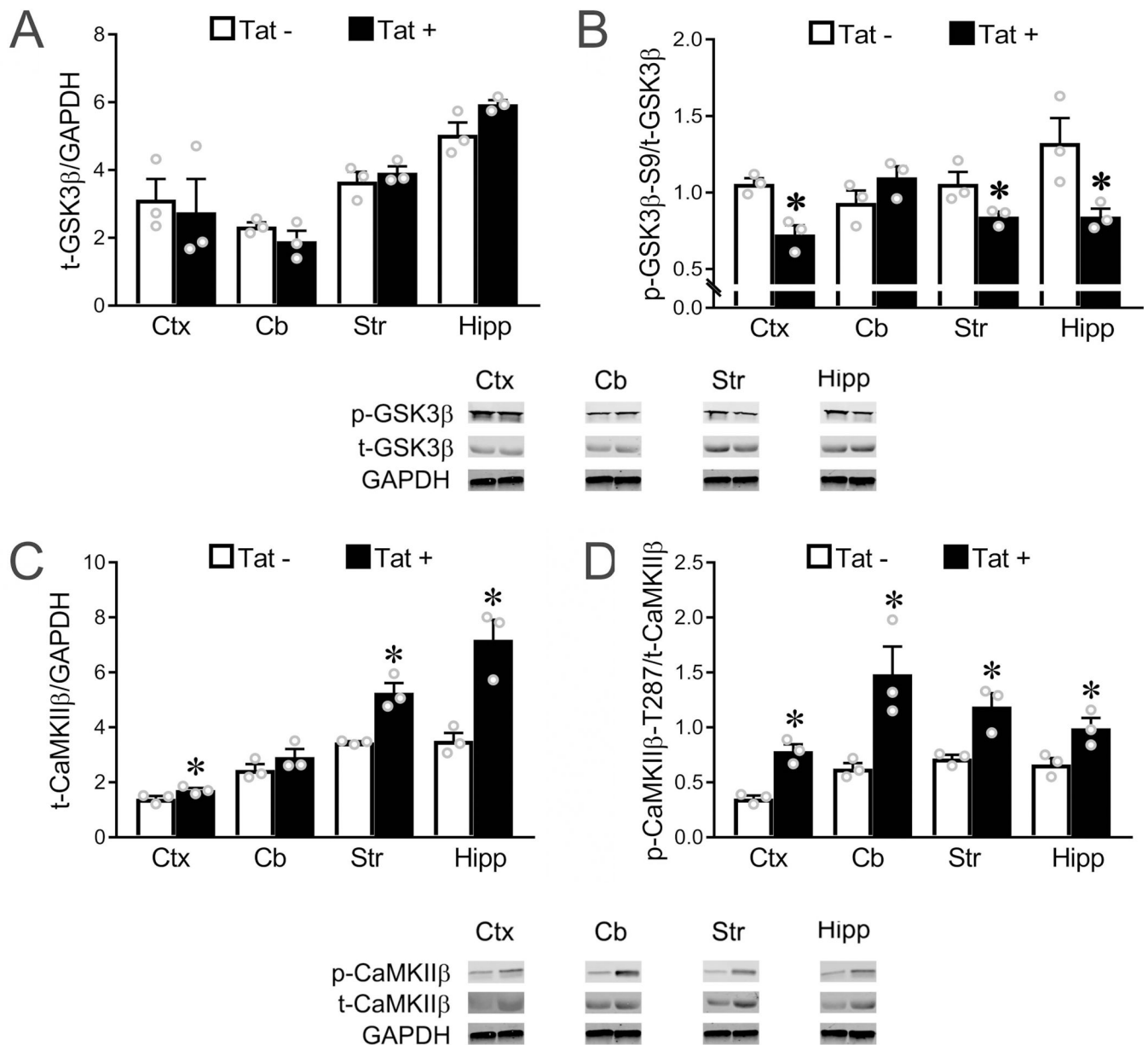


Figure 2. *In vivo* expression of Tat activates GSK3 β and CaMKII β in multiple brain regions. (A) *In vivo* expression of Tat has no effect on total GSK3 β level in any of the four brain regions we examined. (B) *In vivo* Tat expression leads to upregulated activity of GSK3 β (decreased ratio of p-GSK3 β -S9/t-GSK3 β) in cortex, striatum and hippocampus, but not cerebellum ($p = 0.077$). (C-D) Western blots show that Tat expression *in vivo* leads to an increased total CaMKII β level in cortex, striatum, and hippocampus (C, cerebellum: $p = 0.09$), and upregulated CaMKII β activity (increased p-CaMKII β -T287/t-CaMKII β ratio) in all four regions examined (* $p < 0.05$ vs. corresponding control). In A-D, Ctx: cortex; Cb: cerebellum; Str: striatum; Hipp: hippocampus. Data in A-D are based on the results from $N=3$ individual Tat+ and Tat- mice, each from a different litter.

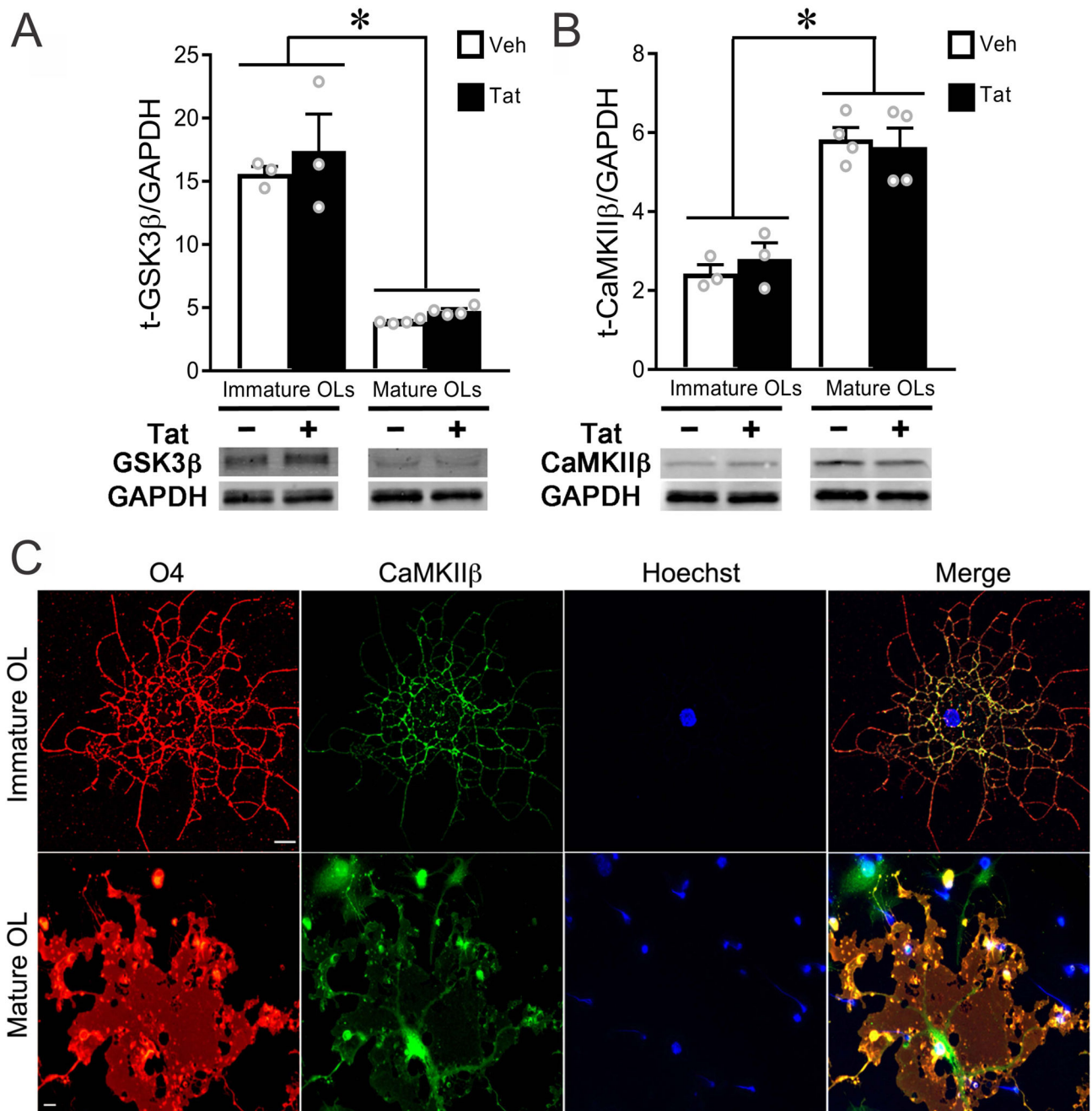


Figure 3. Expression level and localization of CaMKIIβ differ between immature and mature OLs.

(A) Western blotting showed that GSK3β levels were significantly (~3 fold) higher in immature versus mature OLs *in vitro*, and unaffected by Tat at either stage. (B) Expression of CaMKIIβ was significantly lower in immature versus mature OLs *in vitro*, and not affected by 96 h Tat treatment. (C) Confocal images (maximum projection) of fluorescent immunostaining showed that CaMKIIβ was expressed in OLs *in vitro*. In immature OLs, the majority of CaMKIIβ was found in cellular processes. In mature OLs, CaMKIIβ was found

both in cell body cytoplasm and along larger processes. (Scale bar: 10 μm ; *: $p < 0.05$, vs. corresponding control). *Experiments in A and B represent results from $N=3$ (immature) or $N=4$ (mature) independent cultures prepared from mice of different litters.*

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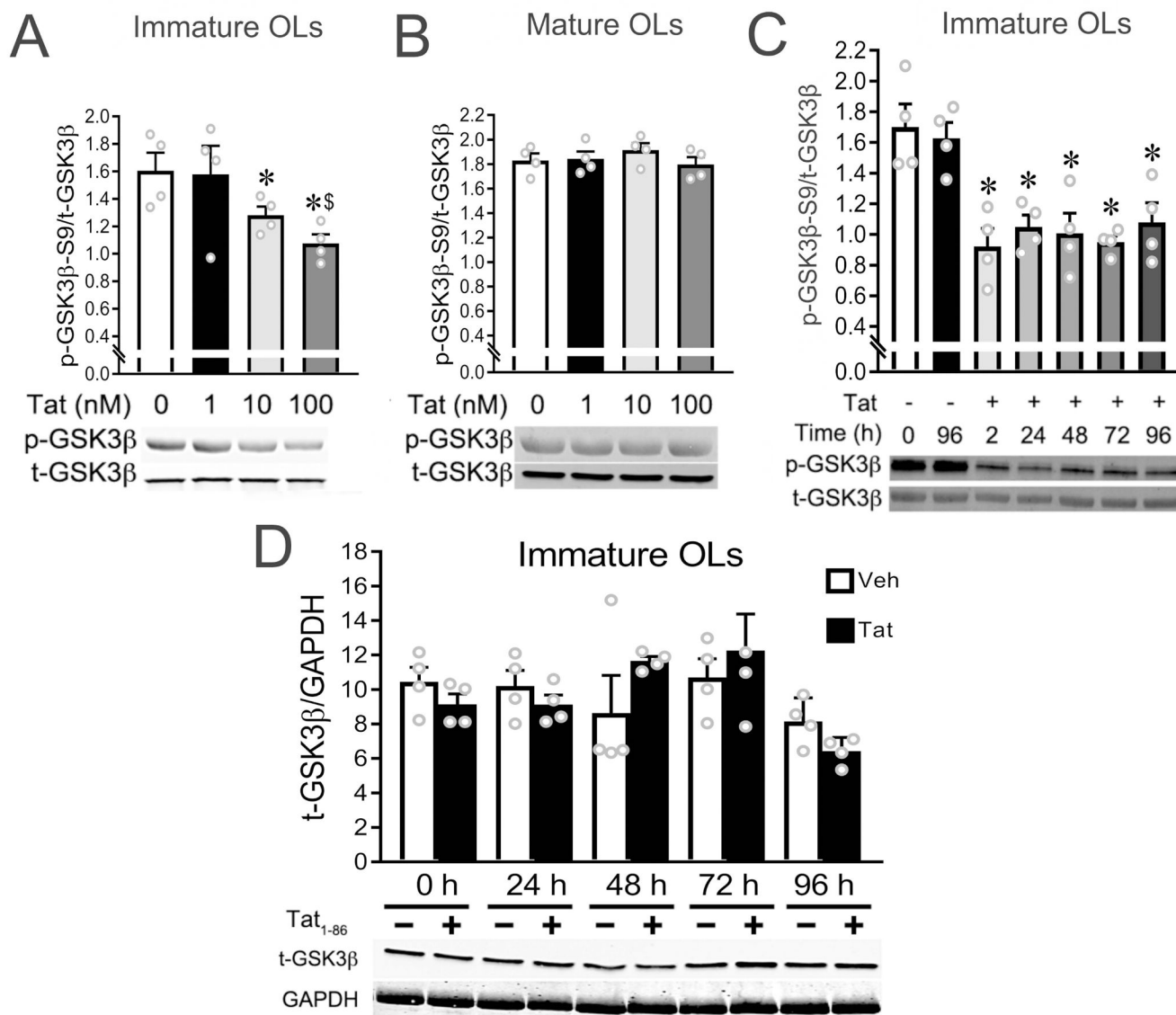


Figure 4. Tat upregulates GSK3 β activity in immature OLs in vitro.

(A) Tat induced a dose-dependent upregulation of GSK3 β activity (decreased ratio of p-GSK3 β -S9/t-GSK3 β) in immature OLs. (B) GSK3 β activity in mature OLs was not affected by Tat. (C) The upregulated GSK3 β activity induced by 100 nM Tat in immature OLs persists over 96 h. (D) Total GSK3 β levels in immature OLs were not affected by 100 nM Tat treatment within 96 h. (* $p < 0.05$ vs. controls lacking Tat; § $p < 0.05$ vs. 10 nM Tat).

Experiments in A–D represent results from $N=4$ independent cultures prepared from mice of different litters.

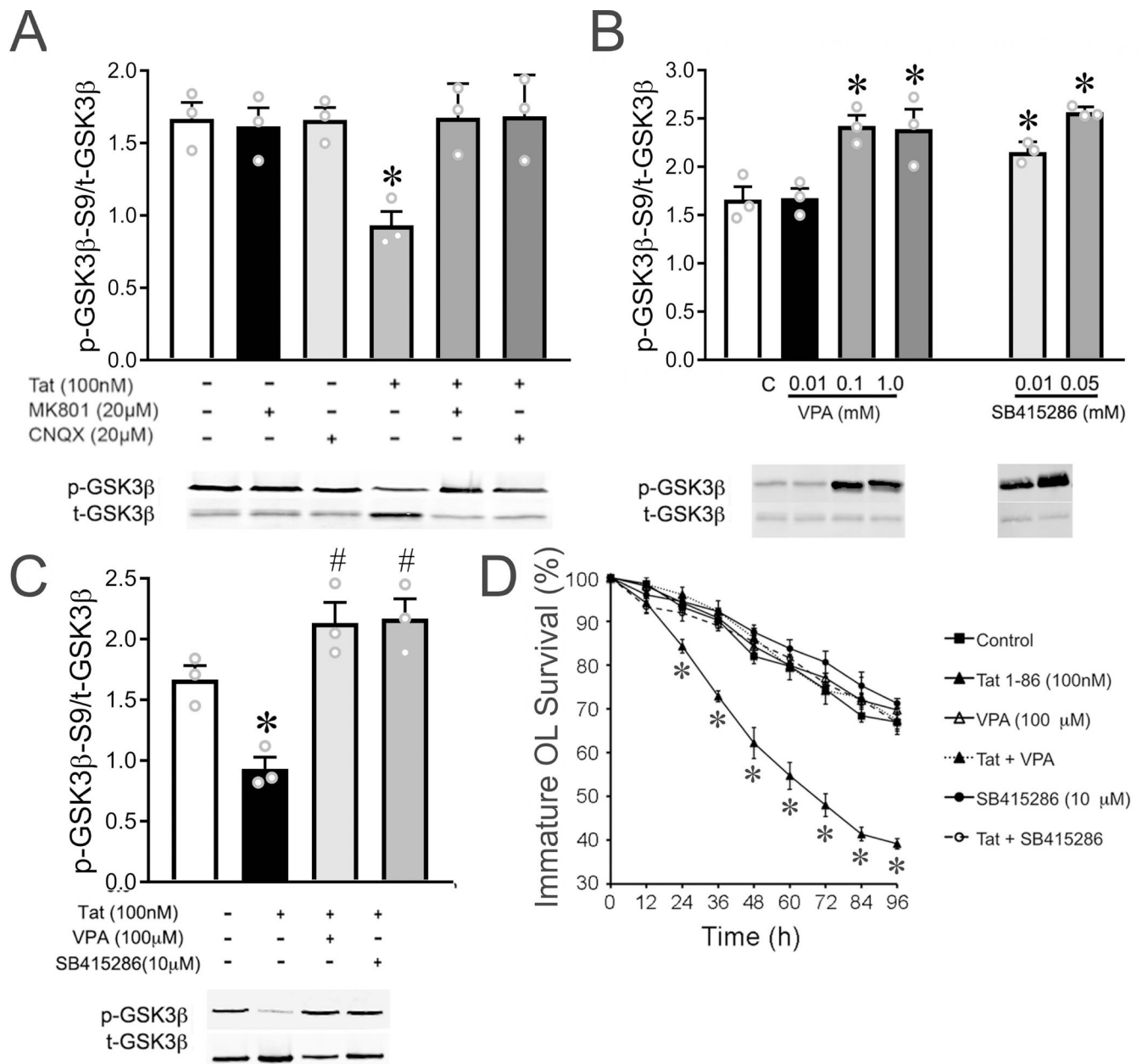


Figure 5. Inhibiting GSK3 β activation rescued immature OLs from Tat-induced death *in vitro*. (A) Both MK801 and CNQX fully reversed the upregulation of GSK3 β activity induced by Tat. (B-C) Both VPA (0.1–10 mM) and SB415286 (0.01–0.05 mM) downregulated basal GSK3 β activity in immature OLs (B), and reversed the elevation of GSK3 β activity caused by 24 h Tat (C). (D) Both VPA (100 μ M) and SB415286 (10 μ M) rescued immature OL death induced by 96 h Tat, although neither VPA nor SB415286 by themselves affected immature OL viability. (*, #: $p < 0.05$ vs. non-treated control.) *Experiments in A–C* represent results from $N=3$ independent cultures prepared from mice of different litters. For each of $N=4$ independent experiments in D, 50 OLs were tracked for every treatment during the course of the entire 96 h time period and survival effects were analyzed by a repeated measures two-way ANOVA.

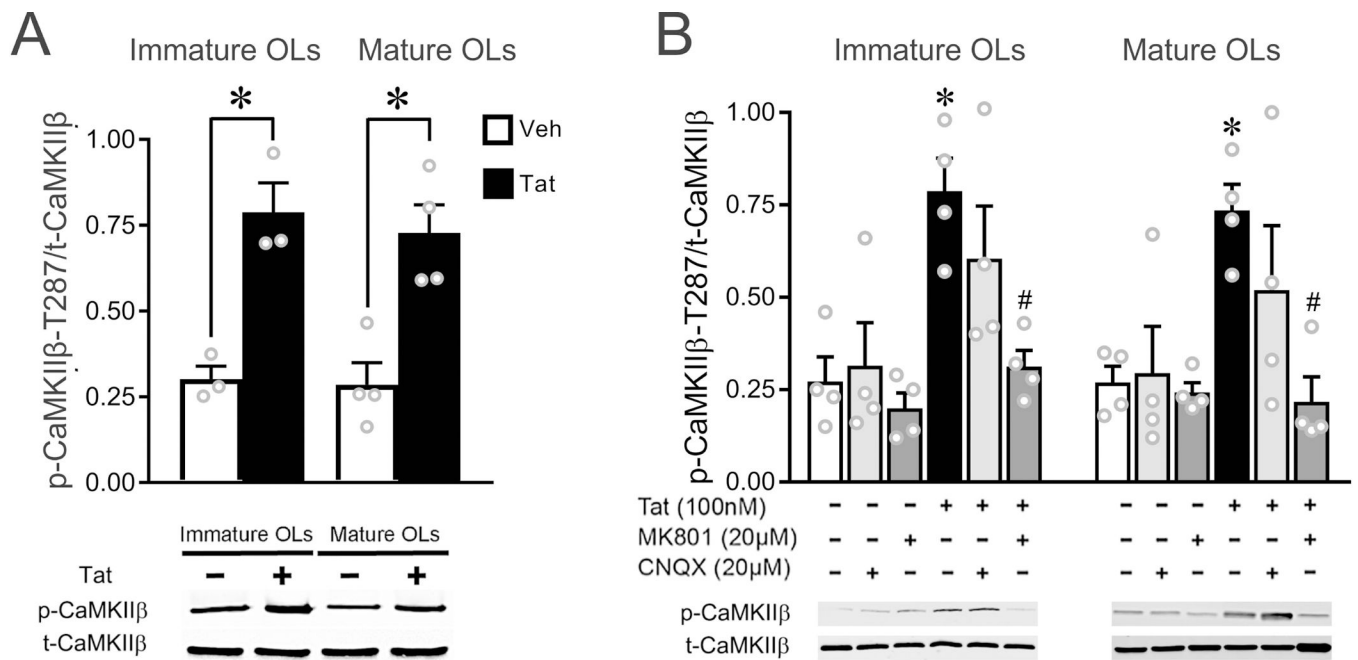


Figure 6. Tat activates OL CaMKIIβ *in vitro* via iGluRs in both immature and mature OLS.

(A) Western blot results indicate that CaMKIIβ activity was significantly upregulated in both immature and mature OLS after 1 h Tat (100 nM) treatment. (B) Tat-induced upregulation of CaMKIIβ activity can be completely inhibited by MK801, and partially reversed by CNQX (not different from either control or Tat; $p=0.113$ vs Tat). (* $p < 0.05$ vs. corresponding vehicle control; # $p < 0.05$ vs 100 nM Tat.) Results represent $N=3$ or $N=4$ independent cultures prepared from mice of different litters.

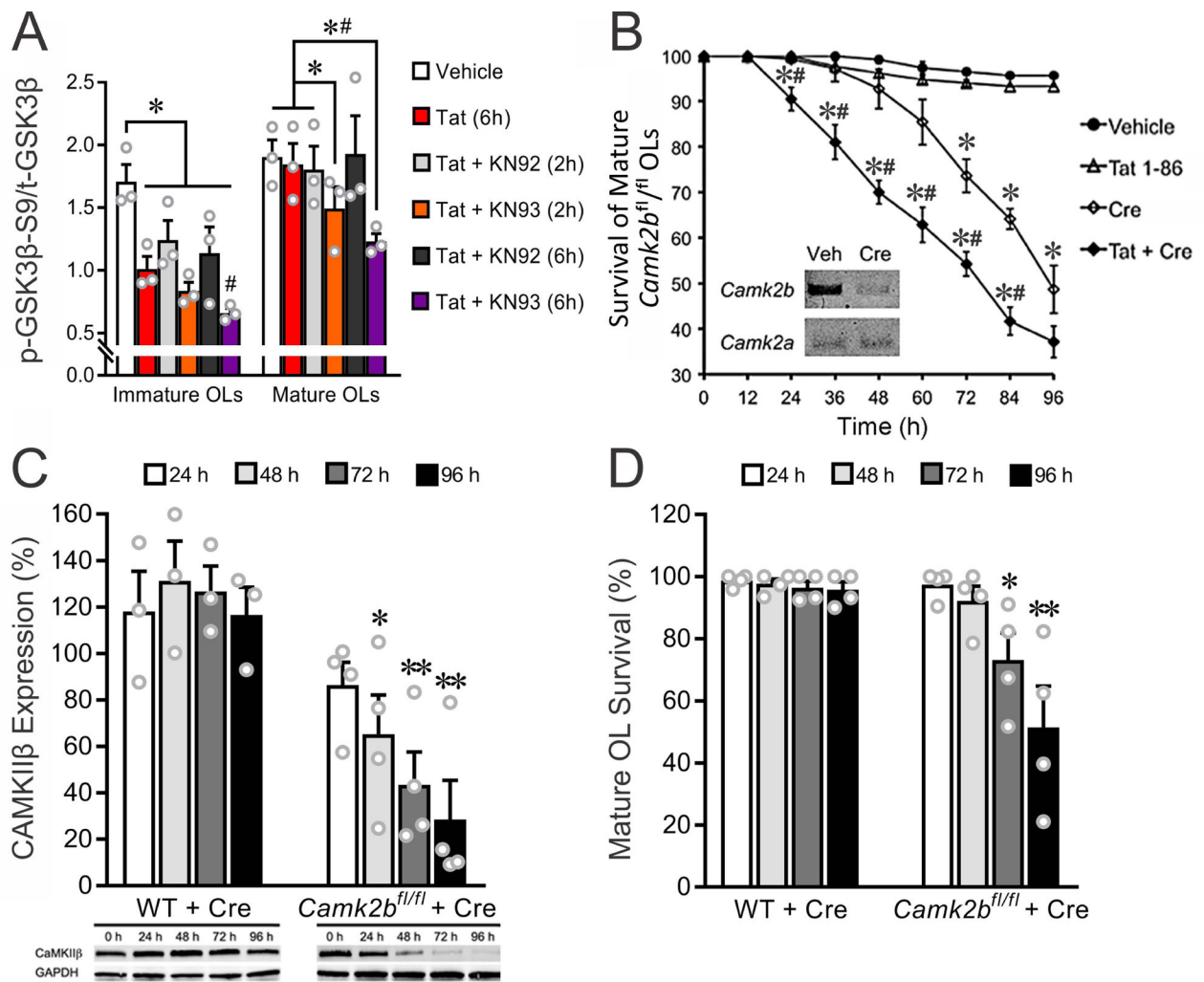


Figure 7. CaMKII β inhibits GSK3 β activity and underlies the survival of mature OLs exposed to Tat.

(A) Inhibiting CaMKII β with KN93 for 2 h and/or 6 h significantly increased Tat (100 nM)-induced GSK3 β activation in cultured OLs. (B) In OLs cultured from *Camk2b^{fl/fl}* mice, 24 h treatment with 50 nM Cre recombinase reduced *Camk2b* (PCR result, inset) and led to increased death in mature OL cultures treated with Tat. (C) Western blotting showed that 50 nM Cre recombinase decreases CaMKII β in mature *Camk2b^{fl/fl}*, but not WT, OL cultures, between 48–96 h. (D) 50 nM Cre recombinase decreases mature *Camk2b^{fl/fl}* OL survival at 72 and 96 h, but does not affect WT OL viability. (A: *· #: $p < 0.05$, vs. corresponding vehicle control or 2 h Tat + KN92, respectively; B: *· #: $p < 0.05$, vs. vehicle control or Cre (50 nM), respectively; C-D: * $p < 0.05$, ** $p < 0.01$, vs. 0 h). Experiments in A, C, and D represent results from $N=3$ or $N=4$ independent cultures prepared from mice of different litters. For each of $N=4$ independent experiments in B, 50 OLs were tracked for every treatment during the course of the entire 96 h time period and survival effects were analyzed by repeated measures, two-way ANOVA.

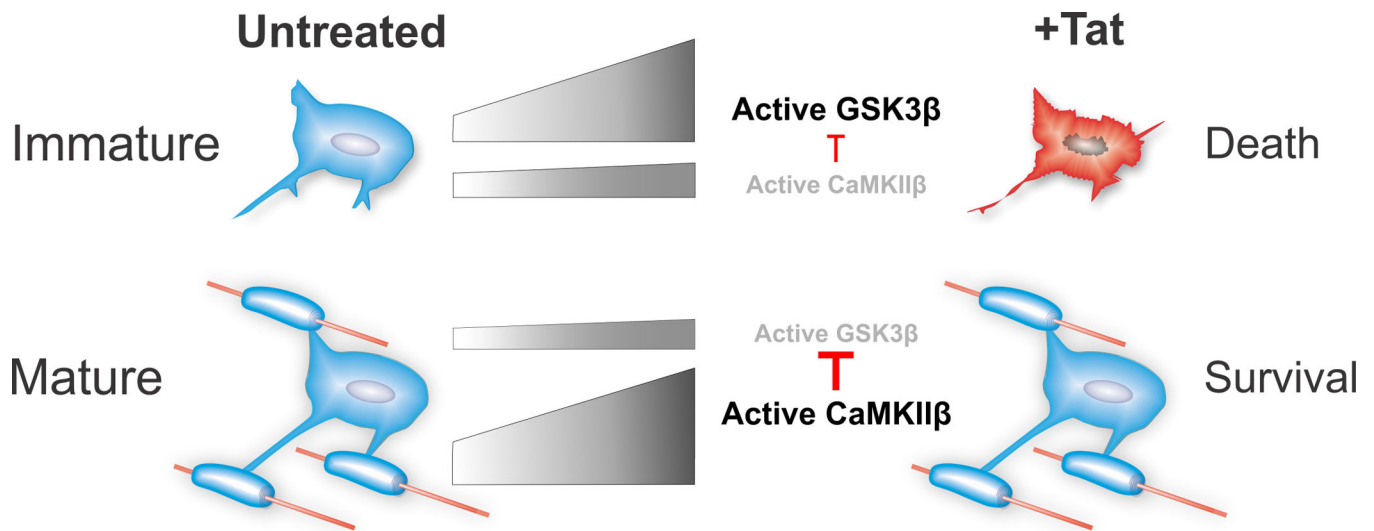


Figure 8. Summary.

HIV-1 Tat-dependent increases in $[Ca^{2+}]_i$ activate both GSK3 β (by dephosphorylating p-GSK3 β at S9) and CaMKII β (by phosphorylating CaMKII β at T287). In mature OLs, large amounts of activated, cytoplasmic CaMKII β inhibit GSK3 β (\perp), which protects OLs from death. Conversely, in immature OLs, higher levels of activated GSK3 β are present. The low levels of activated CaMKII β , which are preferentially distributed within OL cellular processes but not the cell body (\perp), are inadequate to sufficiently inhibit GSK3 β activation. Overactivation of GSK3 β triggers signaling events leading to immature OL death.