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Cell specific loss of kappa-opioid receptors in oligodendrocytes of the dysmyelinating jimpy mouse

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Abstract

Jimpy is a murine mutation in myelin proteolipid protein, leading to premature death of oligodendrocytes and severe central nervous system hypomyelination. Jimpy is a bona fide model of human Pelizaeus-Merzbacher disease. This paper describes a severe reduction in expression of κ-opioid receptors (KOP) in oligodendrocytes of jimpy mice. A cell specific reduction of >90% is apparent by 5 days of age. Expression is not reduced in neurons, and μ-opioid receptor expression is normal. Mechanism(s) leading to deficient KOP expression in jimpy mice remain unclear. We speculate that loss of KOP may be related to increased $[Ca^{2+}]_i$ and premature death of jimpy oligodendrocytes.

Keywords

Proteolipid protein; Pelizaeus-Merzbacher disease; dysmyelination; cell death; corpus callosum; striatum

Introduction

Jimpy is an X-linked mutation in the proteolipid protein (PLP) gene that causes a dysmyelinating phenotype in the central nervous system (CNS) [40, 50]. A single nucleotide change inactivates a splice acceptor site, resulting in excision of exon 5 from PLP mRNA

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[21, 32, 34]. The mutation also causes a frameshift, making the predicted COOH terminus of jimpy PLP completely abnormal. Jimpy is a bona fide model of dominant-negative forms of human Pelizaeus-Merzbacher disease, a rare, inherited, leukodystrophy associated with mutations in, or duplications of, the PLP gene [23, 48]. Jimpy mice produce little CNS myelin, likely due to premature death of oligodendrocytes (OLs) throughout the CNS coincident with active myelination [30]. Arborization, myelin membrane formation, and survival are adversely affected in vivo and in culture. Jimpy mice exhibit tremors by 8–10 days, followed by death at 20–28 days. OLs from jimpy mice show additional abnormalities that appear unrelated to loss of a myelin protein. These include altered pH, Em, $[Ca^{2+}]_i$, metabolic function, cAMP signaling, and proliferation/cell cycle [11, 12, 25, 26, 29, 42]. Many defects occur in situ or in culture long before the cells die, suggesting a direct or indirect contribution to OL death and dysmyelination. We previously showed that jimpy OLs grown in vitro also fail to express κ-opioid receptors (KOP), although most normal OLs express KOP throughout development [28]. The present study examined expression of KOP by jimpy OLs in vivo. Endogenous opioids normally modulate aspects of glial development [15, 16, 20, 28, 36, 38, 44]. Therefore, KOP loss might contribute to abnormalities in OL phenotype and function both in jimpy mice and in human diseases involving PLP mutations.

Methods

Jimpy mice were bred from carrier pairs (B6CBACa *A*$^{w\text{-}J}$/*A-Plp1*^{jp} *Eda*^{Ta}/J) (Jackson Laboratory, Bar Harbor, ME). 16–18 day mutant mice were identified by characteristic tremors. Younger mutants were identified by DdeI restriction analysis after PCR amplification [26]. Mice were anaesthetized by halothane exposure, using procedures to minimize pain outlined in the NIH Guide for Care and Use of Laboratory Animals, then perfused transcardially with 4% Zamboni's fixative. Cerebral hemispheres were postfixed (18h, 4ºC), infiltrated overnight sequentially in 10% and 30% sucrose, embedded in Tissue Tek OCT compound (Sacura Finetek, Torrance, CA) and stored at −80ºC.

Sections (7 μm) were immunostained sequentially for opioid receptors and antibodies specific for either OLs (APC) or neurons (NeuN). Tissue was permeabilized, incubated overnight at 4ºC in polyclonal antibody to either KOP-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or μ-opioid receptor (MOP) (Chemicon, Temecula, CA), followed by monoclonal anti-APC/CC-1 (Oncogene, San Diego, CA) or anti-NeuN (Chemicon). Primary antibodies were visualized using appropriate fluorescent reagents. Sections were stained with Hoechst 33342 to identify nuclei (Molecular Probes), then mounted in ProLong antifade reagent (Molecular Probes).

OLs were quantified in the corpus callosum because of their high abundance in that region. Neurons were quantified in striatum since corpus callosum lacks neuron cell bodies, and a high percentage of striatal neurons express opioid receptors. 100 random OLs or neurons, identified by APC or NeuN, were selected per section at 2 ages. Hoechst staining was assessed, and cells were examined for KOP or MOP staining only if soma were associated with an intact Hoechst-labeled nucleus. Two sections per mouse were examined at 63X magnification for each staining regimen (200 total cells), then averaged as a single N for

statistical purposes, with N=6–9 mice per group. Results were analyzed by ANOVA with Duncan's post-hoc test (Statistica; StatSoft, Tulsa, OK).

KOP levels were also examined by Western blot of corpus callosum using standard procedures [26]. Tissue was homogenized in RIPA buffer with protease inhibitors (Roche, Indianapolis, IN) and protein concentrations determined by BCA Protein Assay (Pierce Chemical Co., Rockford, IL). Samples (5 μg) from 4–6 wild-type and jimpy mice were run on a single 10% Tris-HCl Criterion Precast Gel (Bio-Rad, Hercules, CA), transferred to PVDF membranes (Hybond-P; Amersham Biosciences, Piscataway, NJ), and stained using polyclonal anti-KOP (Santa Cruz) and monoclonal β-actin (Chemicon) antibodies. Blots were visualized with SuperSignal West Femto Substrate (Pierce), scanned and analyzed (Kodak Image Station 440CF). Integrated band volumes from the same gel, corrected for actin loading, were compared using a student's t-test.

Results and Discussion

Tissue from 5–8 day jimpy animals contained a seemingly normal complement of OLs, consistent with reports that immature stages of the lineage are phenotypically normal, and that dysmyelination and OL death occur when OLs begin to produce myelin [13, 30]. Even though cell density and morphology appeared normal, both cell counts (Fig. 1) and immunostaining (Fig. 2) showed a substantial reduction in the percentage of APC^+ OLs expressing KOP in 5–8 day corpus callosum. While 67.4% of OLs in wild-type tissue expressed detectable KOP, this was reduced to 4.5% in jimpy. The percent of OLs expressing KOP normally increased with age (Fig. 1). Instead, the discrepancy in KOP staining in jimpy at 16–18 days remained highly significant (85.1% in wild-type vs. 8.8% in jimpy; Fig. 1A), thus ruling out the explanation of a temporal delay in receptor expression. Later ages were not examined since jimpy mice die prematurely. Although corpus callosum was the only region where MOP and KOP were both quantified, OLs throughout the brain were KOP deficient. For example, APC+/KOP+ cells were reduced over 90% at both ages in striatum.

To test for a general defect in opioid receptors in jimpy OLs, we examined expression of MOP, which are expressed on large numbers of normal OLs both *in vivo* and in culture [28, 44]. Figure 1B shows no difference in MOP expression in wild-type versus jimpy OLs at either 5–8 days (59.8% vs. 51.1%) or 16–18 days (58.9% vs. 53.2%). MOP expression on normal OLs was somewhat reduced or delayed from in vitro findings [28] perhaps reflecting different milieus, or regional variations in timing of receptor expression. We also tested whether reduced KOP expression was specific to OLs by examining KOP expression in striatal neurons. Fig. 3 shows a significant increase in the percent of KOP+ neurons with age in both normal and jimpy striatum. There was no difference due to genotype at either age. Overall, the results indicate a severe reduction of KOP expression in jimpy CNS that is specific to OLs and not accompanied by decreased MOP.

Immunoblots from 5–8 day corpus callosum always showed a significant decrease in KOP (Fig. 4), mirroring the immunostaining. Results from 16–18 day samples were variable. A total of 3 sets of blots from 16–18 day old mice were performed, comparing "N"s of 4–6

jimpy and wild-type samples each. Only one experiment was significant. Since immunoblots reflect the aggregate expression on all KOP-expressing cells, which include astroglia and microglia as well as neurons and OLs, immunostaining likely provides a more accurate picture of KOP changes in OLs alone. Additionally, the jimpy CNS exhibits astrogliosis throughout the lifespan [43] and KOP expression in hypertrophied astroglia may offset KOP loss in the OL population

Mechanism(s) underlying reduced KOP expression in jimpy mice are unclear, although maintained MOP expression argues against a general decline of opioid signaling in sick/dying jimpy OLs. The finding that KOP expression is significantly reduced by day 5 also counters this argument, since OLs in jimpy corpus callosum appear phenotypically normal and are not dying at that age [30]. As KOP levels are unaffected in neurons, the mutation appears specific for OLs. Ultimately, whether diminished KOP levels result from failure of production or failure to be inserted into OL membranes, they must stem from either loss of normal PLP or production of mutant jimpy PLP. One intriguing possibility comes from work showing that both PLP and its alternative splice product DM-20, bind cholesterol and are components of lipid rafts [41]. Missense PLP mutations similar to jimpy (jimpy-msd and rumpshaker) lead to impaired cholesterol binding and lipid raft association [31]. Lipid rafts can influence receptor function and turnover, including trafficking, stability, and internalization [1], which might in turn affect expression of KOP and other G-protein coupled receptors associated with lipid rafts [35, 51].

What are possible ramifications of reduced KOP levels in jimpy OLs? Overall, little is known about specific actions of KOP agonists on OL function or survival. In vivo studies generally suggest that selective KOP signaling is protective against CNS damage due to ischemia or trauma [3–5, 17, 18]. Much of this effect is probably indirect, mediated by reduced production of inflammatory agents or vascular changes [18, 37]. However, direct protective effects of KOP signaling may occur. Several studies with KOP agonists in vivo have shown specific sparing of white matter, suggesting direct or indirect effects on OLs [4, 18]. If direct protective effects occur through KOP signaling, loss of KOP might make jimpy OLs more vulnerable to injury. Our culture studies showed protective KOP signaling against glutamate toxicity in OLs [27], while KOP signaling has been variably effective against glutamate toxicity in neuron cell lines and cortical cultures [6, 7]. Studies discussed above use specific KOP agonists and antagonists. They do not cite studies with dynorphin peptides, whose interpretation is complex due to conflicting glutamatergic and KOP activities, as well as non-opioid effects [10, 14, 19, 46, 47, 49]. Taken together, discrepancies between in vivo and in vitro results suggest KOP signaling effects are cell and context specific, although the sparse data on hand suggests that KOP signaling may protect OLs. Supporting our concept that opioid status can affect myelination, the KOP antagonist and partial MOP agonist buprenorphine was recently shown to have multiple and complex actions on myelin and OLs during CNS development [39].

We previously showed increased baseline $[Ca^{2+}]_i$ in jimpy OLs [26], and it is tempting to speculate a role for KOP since KOP-mediated signaling is implicated in regulating $[Ca^{2+}]_i$. Specific KOP agonists inhibit Ca^{2+} influx or increase extrusion in neurons [2, 7, 8, 33], and this might play a role in CNS protection observed with KOP signaling. KOP loss on jimpy

OLs might thus elevate $[Ca^{2+}]_i$. Astroglia, in contrast, respond to KOP activation with increased $[Ca^{2+}]_i$, both through influx (L-type channels) and mobilization of intracellular stores [9, 16]. Another intriguing possibility is that loss of KOP-mediated protection evokes autocrine effects that elevate $[Ca^{2+}]_i$ in jimpy OLs. OLs synthesize several opioids, including the KOP agonist dynorphin, which is detected even in immature OLs [27]. Some dynorphin peptides have excitotoxic, non-opioid activities via direct cell membrane effects or through interaction with glutamate receptors (reviewed in [19]) [22, 46, 47], including *N*-methyl-D-aspartate receptors, which are expressed by OLs [45]. If KOPs were reduced, the predominant dynorphin effects on OLs might be excitotoxicity and calcium influx, perhaps contributing to elevated $[Ca^{2+}]_i$ and premature death. Alternatively, KOP can activate ERK in neural precursors, including those giving rise to OLs [24]. If KOP couples to protective ERK pathways in OLs, diminished KOP signaling might contribute to excitotoxic injury and/or a loss of ERK-mediated protection, promoting premature OL death.

In conclusion, jimpy mice exhibit a profound reduction in KOP expression in the CNS that is specific for OLs. Numbers of OLs expressing KOP are dramatically reduced by 5 days, and remain so throughout life. Jimpy OLs die prematurely, before forming myelin. Although reduced KOP levels probably do not directly cause OL death, we speculate that loss of KOP may increase vulnerability of jimpy OLs to other toxic events, perhaps by modulating $[Ca^{2+}]_i$ or through loss of protective KOP-mediated signaling effects.

Acknowledgments

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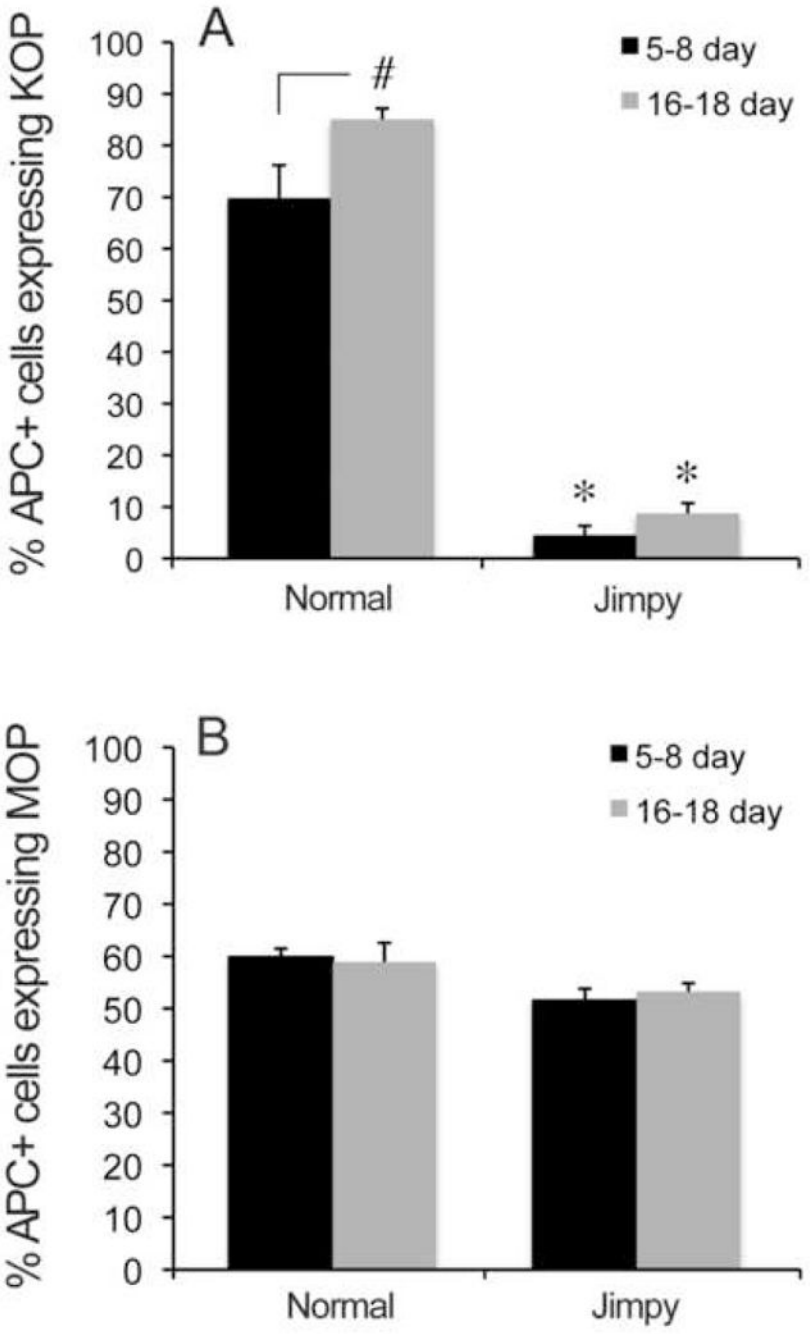

Figure 1.

Expression of KOP and MOP in normal and jimpy (Jp) OLs. A. Most normal, APC^+ OLs at 5–8 and 16–18 days express KOP. In contrast, <10% of jimpy OLs immunostain for KOP at either age (*$p<0.0001$ vs. normal, same age). KOP expression increases with age in normal but not jimpy OLs (# $p<0.01$). B. Unlike the situation for KOP, MOP expression was apparently not influenced by either genotype or age. Error bars represent S.E.M.

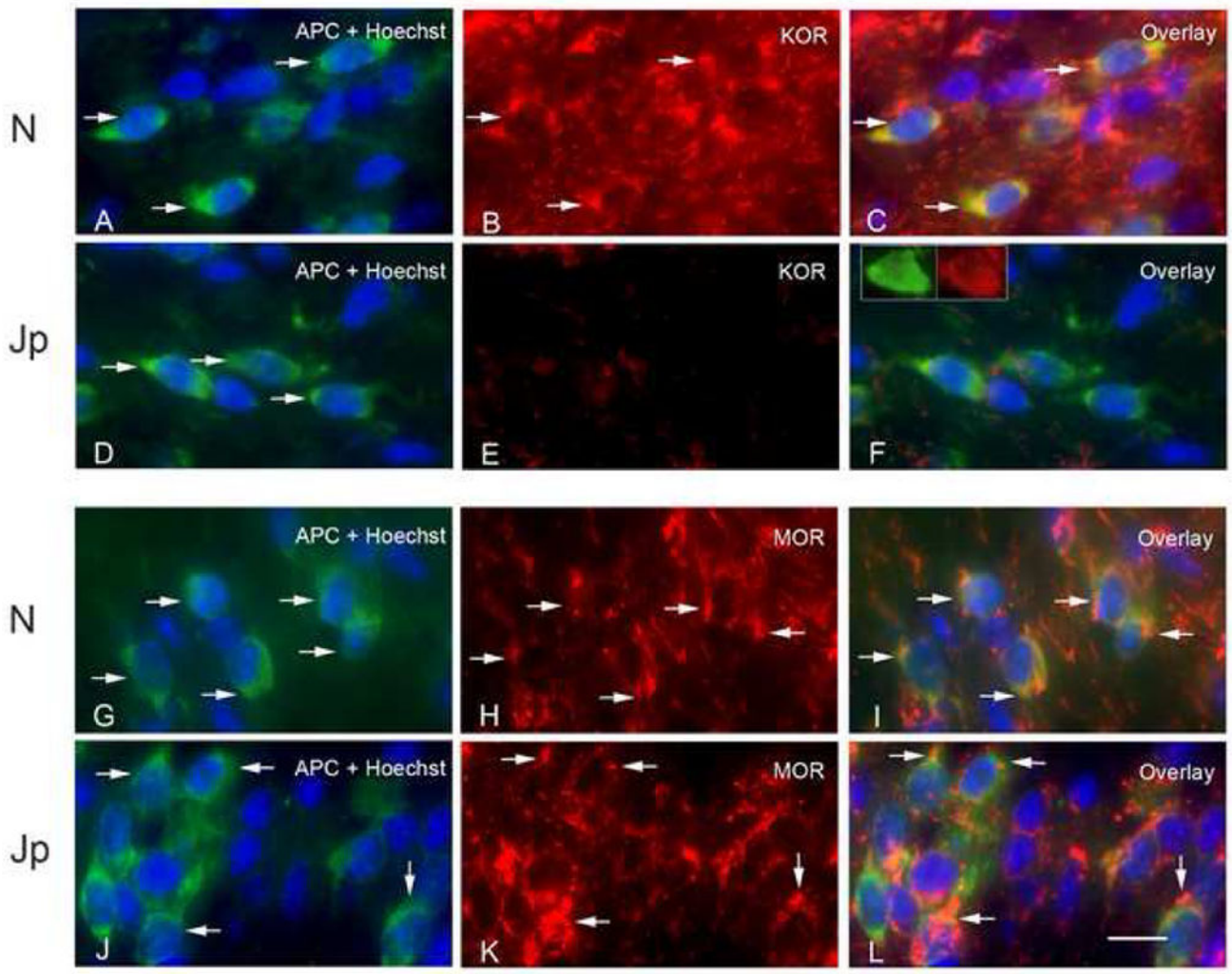

Figure 2.

OLs from 16 day normal and jimpy (Jp) corpus callosum double-labeled for APC and either KOP (A–F) or MOP (G–L) and counterstained with Hoechst 33342. Many normal, APC^+ OLs express KOP robustly (arrows indicate double-labeled cells in panels A–C). There is little KOP staining in jimpy corpus callosum (D–F). Although APC^+ OLs are present (arrows in D), most are not KOP+ (E). The inset in F shows an APC+, KOP+ jimpy OL to illustrate morphology. In contrast, there is robust expression of MOP in APC^+ OLs in both normal (G–I) and Jp (J–L) corpus callosum. Arrows in G–I indicate double-labeled cells in wild-type. Arrows in J–L indicate double-labeled cells in jimpy.

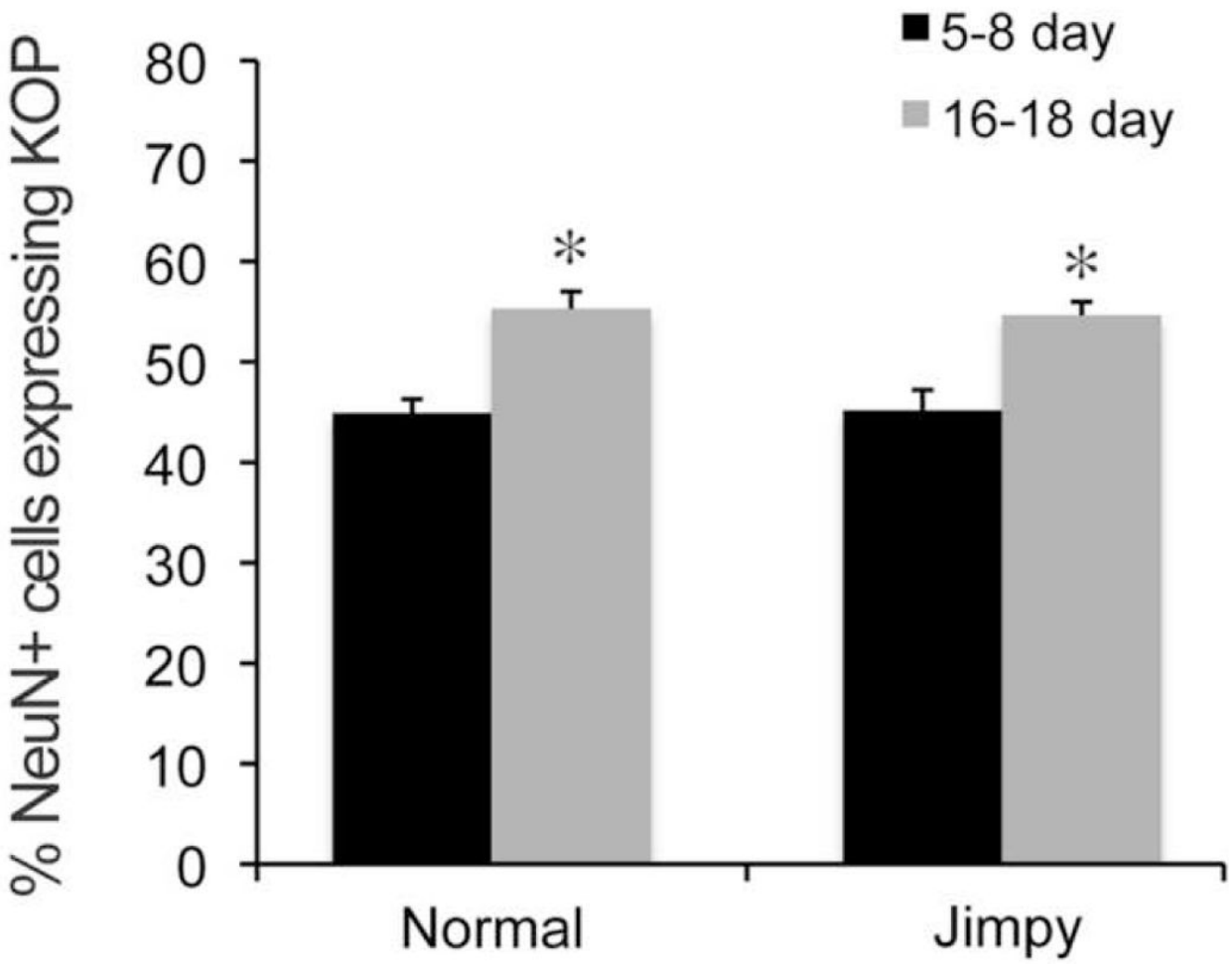

Figure 3.

KOP expression in striatal neurons. KOP expression increases with age in both genotypes. There is no difference in the percentage of KOP-immunoreactive striatal neurons in jimpy as compared to wild-type mice at either 5–8 or 16–18 days. * $p<0.005$ vs. same genotype at 5–8 days of age. Error bars represent S.E.M.

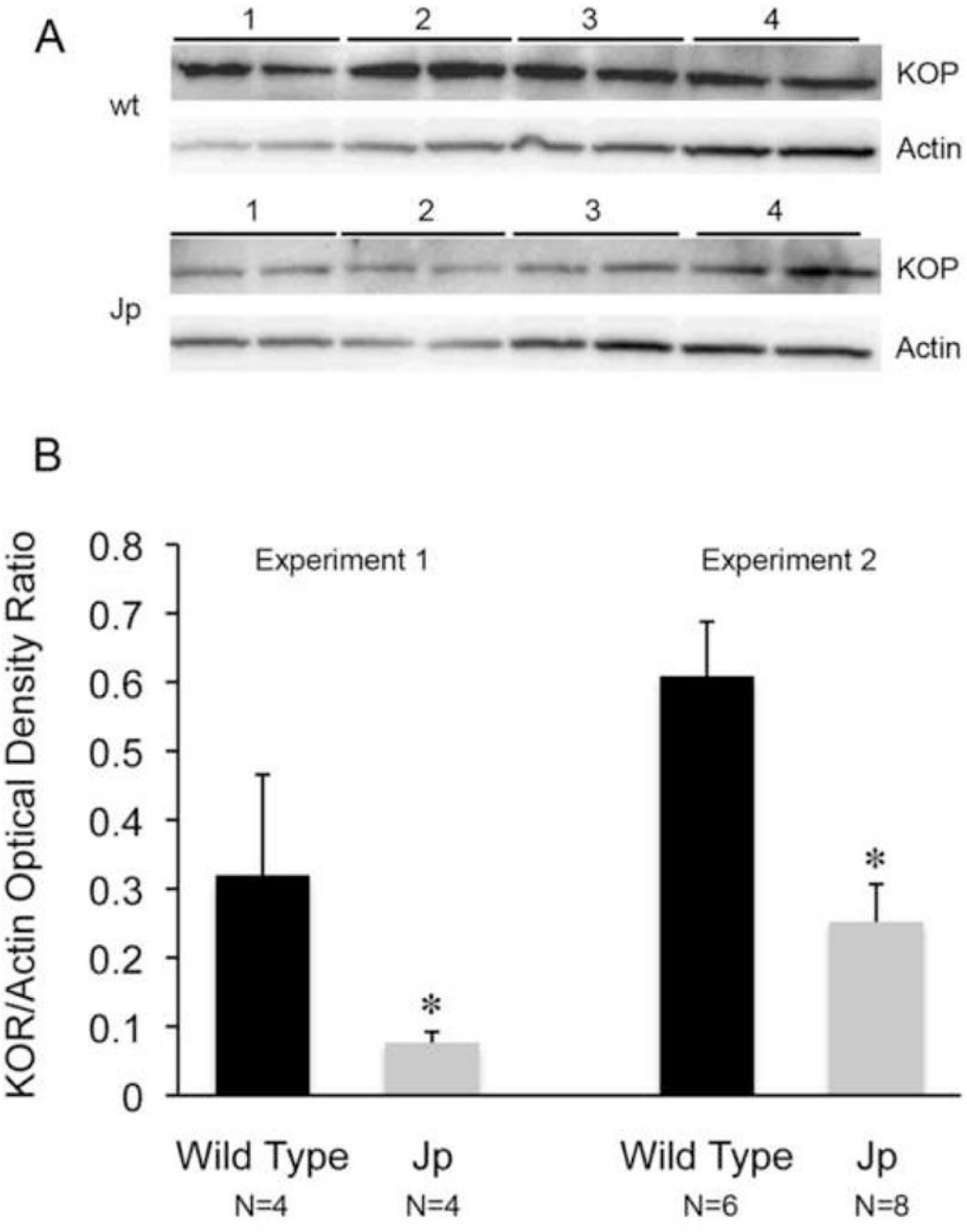

Figure 4.

Immunoblot analysis of KOP in corpus callosum at 5–8 days. Panel A shows a representative immunoblot with duplicates of 4 wild-type (wt) and jimpy (Jp) samples stained for KOP and actin. KOP staining revealed a single band at 55 Kd. Panel B shows results of 2 separate experiments using different mice. Although absolute values differ between experiments, the jimpy KOR/actin ratio is significantly decreased from normal in

both (* $p<0.02$ in experiment 1; * $p<0.01$ in experiment 2). Data in Experiment 1 are from panel A immunoblot. Error bars represent S.E.M.