Adaptron-associated kinase 1 (AAK1), a member of the Arl1/Prk1 family of serine/threonine kinases, plays a role in modulating clathrin coated endocytosis of specific surface receptors. We have demonstrated that AAK1 inhibitors are efficacious in rodent models of neuropathic pain (Kostich et al., 2016). Here we have characterized the binding properties and distribution pattern of the tritiated AAK1 radioligand, [3H]BMT-046091, in rodents and cynomolgus monkeys, and used the radioligand to measure the brain target occupancy following systemic administration of AAK1 inhibitors. We have found that [3H]BMT-046091 is potent and selective AAK1 inhibitor. It inhibits AAK1 phosphorylation of a peptide derived from a physiologic substrate, the m2 subunit of the adaptor protein complex, with an IC50 value of 2.8 nM, and is inactive at >5 μM in a panel of functional or binding assays for receptors, transporters and enzymes. [3H]BMT-046091 binding in the brain is absent in the AAK1 knockout mouse, and is displaceable with a high concentration of AAK1 inhibitors in wild type mice. Specific [3H]BMT-046091 binding is widespread in the brain and spinal cord with the highest density in the cortex, hippocampus, amygdala, striatum and thalamus. In the spinal cord, [3H]BMT-046091 binding appears enriched in the dorsal horn superficial layers. Oral administration of LP-935509, an AAK1 inhibitor, results in a dose-dependent occupation of AAK1 binding sites in the brain and spinal cord. The increase in AAK1 binding site occupancy by LP-935509 correlates with the decrease in antinociceptive responses in the rat chronic constriction injury model of neuropathic pain.

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1. Introduction

Adaptron-associated kinase 1 (AAK1), a member of Arl1/Prk1 family of serine/threonine kinases, is involved in the endocytic pathway (Smythe and Ayscough, 2003). AAK1 is an interacting partner of the adaptor protein (AP2) complex and specifically interacts with the σ-adaptin subunit of AP2 and phosphorylates the μ2 subunit of the AP2 complex (Conner and Schmid, 2002). The μ2 subunit binds specific tyrosine motifs of certain membrane receptors and mediates cargo recruitment to clathrin-coated vesicles (Ohno et al., 1995). Phosphorylation of the μ2 subunit by AAK1 promotes a high affinity interaction between the μ2 subunit and cargo with the with the specific tyrosine motif (YXXPhi) thereby playing a role in maximizing the efficiency of receptor internalization (Henderson and Conner, 2007). Clathrin-coated vesicle formation is also needed for recycling of synaptic vesicles particularly after neurotransmission. Although the AP2 complex is important in synaptic vesicle recycling, preventing μ2 phosphorylation through the use of a threonine 156 to alanine replacement does not seem to impact synaptic recycling (Kim and Ryan, 2009).

While AAK1 has mostly been studied in the context of regulation of receptor endocytosis, several studies have suggested AAK1 may be linked in some manner to psychiatric and neurological...
disorders. Analysis of postmortem cerebellum in schizophrenic patients shows that many genes involved in presynaptic vesicular transport including AAK1 are upregulated (Mudge et al., 2008). Single nucleotide polymorphism in an intron of the AAK1 gene has been linked to the onset of Parkinson’s disease (Latourelle et al., 2009). AAK1 protein is decreased in the spinal cord of amyotrophic lateral sclerosis (ALS) patients. In rodent models of ALS, AAK1 is found to be mislocated with mutant superoxide dismutase 1 (SOD1) proteins (Shi et al., 2014). Our recent study has revealed an important role of AAK1 in neuropathic pain and anti-neuropathic pain effects of AAK1 inhibition in rodents (Kostich et al., 2016). We have found that AAK1 knockout mice exhibit a reduced response in the persistent pain stage of the formalin model and fail to develop tactile allodynia in a spinal nerve ligation (SNL) model of neuropathic pain. The anti-neuropathic pain effects can be reproduced using small-molecule AAK1 inhibitors (Kostich et al., 2016). Furthermore, AAK1 in the spinal cord appears to be critically important in mediating the antineuropathic pain effects as local administration of AAK1 inhibitors causes reduced nociceptive behavioral responses and inhibits spontaneous neural activity in the spinal cord in the chronic constriction injury (CCI) model of neuropathic pain (Kostich et al., 2016).

Currently there is little information on AAK1 distribution in the brain and spinal cord. There are also no tools to directly relate the binding of selective inhibitors of AAK1 to observed efficacy in animal models. Thus, in the present study we sought to synthesize a tritiated AAK1 inhibitor as a radioligand to map the distribution of AAK1 binding sites in the brain and spinal cord in rodents and cynomolgus monkeys. We then developed an ex vivo radioligand binding assay to measure the AAK1 target occupancy in the brain and spinal cord following systemic administration of an AAK1 inhibitor and to correlate the target occupancy with the antineuropathic effects of the compound in order to understand the pharmacokinetic-pharmacodynamic relationship.

2. Materials and methods

2.1. Materials

AAK1 inhibitors were synthesized by Neuroscience Discovery Chemistry at Bristol-Myers Squibb (BMS). PK 11195 and gabapentin were purchased from Sigma (St. Louis, Mo). [3H]JBTM-046091 (specific activity, 84 Ci/mmol; concentration, 1 mCi/ml; purity, 99%) was synthesized by Radiochemistry Group at BMS. [3H]PK 11195 (NET885) and [3H]Gabapentin (NET1182) were purchased from Perkin Elmer (Waltham, MA).

2.2. Animals and tissue collection

As reported previously (Kostich et al., 2016), AAK1 wild-type and knockout mice were generated on a C57 × 129 hybrid background strain. Sprague-Dawley rats were purchased from Charles River Laboratories. All animals were housed in groups of 2–4 in colony rooms maintained at a constant temperature (21 ± 2 °C) and humidity (50 ± 10%). The rooms were illuminated 12 h per day (lights on at 0600 h). Animals had ad libitum access to food (rodent diet; PMI Nutrition International, Brentwood, MO) and water throughout the studies. Animals were maintained in accordance with the guidelines provided by the Animal Care and Use Committee of Bristol-Myers Squibb. Mouse and rats were sacrificed by decapitation, and the brain and spinal cord were collected. Snap-frozen in dry ice-cold methylpentane citrate and stored at −80 °C until use. Fresh frozen naive cynomolgus monkey brain tissues were purchased from the Analytic Biological Services Company (Wilmington, DE).

2.3. AAK1 kinase assay

Generation of recombinant GST-Xa-hAAK1 (amino acids 30–330) for an AAK1 kinase assay has been described previously (Kostich et al., 2016). Recombinant GST-Xa-hAAK1 (3.5 nM) was then incubated with varying concentrations of AAK1 inhibitors (from 2 μM to 33 pM) in the reaction solution (30 μl in volume) containing 10 mM Tris-HCl pH 7.4, 10 mM MgCl2, 0.1% Tween-20, 1.0 mM DTT, 22 μM ATP, 1.5 μM (5-FAM)-Aha-KEEQSQITSQVTG-QIGWR-NH2, 1.6% DMSO. Kinase reactions were incubated at room temperature for 3 h and terminated by adding 60 μl of 35 mM EDTA buffer to each sample. Reaction solutions were analyzed on the Caliper LabChip 3000 (Caliper, Hopkinton, MA) by electrophoretic separation of the fluorescent substrate and phosphorylated product. IC50 values were derived by non-linear regression analysis of the concentration response data.

2.4. In vitro [3H]JBTM-046091 binding autoradiography

Frozen brains of mice, rats and monkeys, and frozen spinal cord of rats (naïve or CCI) were cut into 20 μm sections on a Cryostat (Thermal Scientific). The sections were mounted on superfrost slides (VWR International, Woburn, MA) and stored at −80 °C until use. On the day of the experiment, sets of slides were incubated with 10 nM [3H]JBTM-046091 in assay solution containing 20 mM Tris-HCl (pH 7.4), 5 mM MgCl2, and 0.1% BSA for 60 min at 22 °C. Non-specific binding was defined by incubation of adjacent sections with 10 nM [3H]JBTM-046091 in the presence of 10 μM BMS-901715 (a potent structurally distinct ligand disclosed in our previous publication that interacts with the same binding site (Kostich et al., 2016)). After incubation, slides were washed in ice cold assay buffer and subsequently dried under a stream of cold air. The slides were then placed in cassettes against [3H]-sensitive storage phosphor-imaging screens (PerkinElmer, Waltham, MA) for 3–7 days, and the screens were then scanned with a Cyclone storage phosphor imaging system (PerkinElmer, Waltham, MA). Captured images were analyzed with OptiQuant Acquisition and Analysis software (PerkinElmer, Waltham, MA). Radioligand binding in a given brain region was measured as digital light units per millimeter squared (DLU/mm2), and the specific binding density was calculated by subtraction of non-specific from total binding values.

In saturation binding studies, adjacent sections were incubated with varying concentrations of [3H]JBTM-046091 from 0.1 nM to 10 nM at 22 °C for 60 min. After washing and drying, the slides plus [3H] standards (American Radiolabeled Chemicals, St. Louis, MO) were placed in cassettes against [3H]-sensitive storage phosphor-imaging screens for 3–7 days, and were then processed as described above for Cyclone storage phosphor imaging acquisition and analysis. Binding signal measured as DLU/mm2 was converted to pM/g for Ki and Bmax calculation.

2.5. In vitro [3H]PK 11195 and [3H]gabapentin binding autoradiography

In vitro [3H]PK 11195 and [3H]gabapentin binding autoradiography procedures were modified from elsewhere (Thurlow et al., 1996; Miller et al., 2013). In brief, for [3H]PK 11195 binding, spinal cord lumbar sections collected from CCI (see below for description of CCI surgery) were incubated with 3 nM [3H]PK11195 in assay solution containing 50 mM Tris-HCl buffer (pH 7.0) at 4 °C for 2 h. The non-specific binding was defined in the adjacent sections incubated in the presence of 10 μM PK11195. For [3H]gabapentin binding, spinal cord lumbar sections collected from CCI or sham-operated rats were incubated with 20 nM [3H]gabapentin in assay solution containing 50 mM Tris-HCl buffer (pH 7.0) at 22 °C
for 30 min. The non-specific binding for $[^{3}H]$gabapentin was defined in the adjacent sections incubated in the presence of 25 $\mu$M gabapentin. After incubation, slides were processed as described above for Cyclone storage phosphor imaging acquisition and analysis.

2.6. AAK1 site occupancy measured using ex vivo $[^{3}H]$BMT-046091 binding autoradiography

In studies to measure the binding site occupancy, LP-935509 was formulated in 10% Cremophor in water, and dosed in naive rats at 1, 3 and 10 mg/kg po. The brain and spinal cord of the rats were collected 2 h post-dose and frozen sectioned, and the sections were then processed for ex vivo binding. In a separate study, LP-935509 was dosed at 1, 3, 10 and 30 mg/kg po in CCI rats (2–3 weeks post-surgery), and the rats were then subjected to a 3 h thermal hyperalgesia test (see below for detail). Thirty minutes after the behavioral test rats $(n=4)$ were decapitated, and the brain tissues were collected and sectioned for ex vivo occupancy assessment. The sections were incubated with 10 nM $[^{3}H]$BMT-046091 in assay solution containing 20 mM Tris–HCl, 5 mM MgCl$_2$ and 0.1% BSA in the absence and presence of 10 $\mu$M BMS-901715 (to define non-specific binding) for 2 min at 22 °C. After incubation, slides were processed as described above for Cyclone storage phosphor imaging acquisition and analysis.

2.7. Chronic constriction injury (CCI) surgery and thermal hyperalgesia measurement in rats

CCI surgery and thermal hyperalgesia measurement were adopted from that described by Kim and Chung (1992) and Ye et al. (2015). In brief, male Sprague-Dawley rats were transferred from the holding area to an aseptic surgery suit. Animals were anesthetized with 2% isoflurane, placed in the prone position and the left hind limb supported and immobilized with adhesive tape. Following shaving of the fur and disinfection of the surgical site and the holding area to an aseptic surgery suit. Animals were anesthetized and tracheotomized. Animals were initially housed individually and then pair-housed 3 days after surgery until behavioral testing. CCI rats were then subjected to a 3 h thermal hyperalgesia test (see below for detail). Thirty minutes after the behavioral test rats $(n=4)$ were decapitated, and the brain tissues were collected and sectioned for ex vivo occupancy assessment. The sections were incubated with 10 nM $[^{3}H]$BMT-046091 in assay solution containing 20 mM Tris–HCl, 5 mM MgCl$_2$ and 0.1% BSA in the absence and presence of 10 $\mu$M BMS-901715 (to define non-specific binding) for 2 min at 22 °C. After incubation, slides were processed as described above for Cyclone storage phosphor imaging acquisition and analysis.

2.8. Data analysis

For radioligand binding autoradiographic studies, binding density in a defined brain region was measured as digital light units per millimeter squared (DLU/mm$^2$). The specific binding was calculated by subtracting the value of the non-specific binding from that of the total binding in each sample. For the occupancy studies, % occupancy was calculated as (1–specific binding in drug treated/specific binding in vehicle treated) × 100%. Nonlinear regression was performed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA). All data were reported as the mean ± SEM. Quantitative data were analyzed using two-way RM ANOVA, followed by Bonferroni post tests or Student’s-t-test as appropriate. The significance level was set at $p < 0.05$.

3. Results

3.1. Characterization of $[^{3}H]$BMT-046091

BMT-046091 is a potent and selective functional inhibitor of AAK1. It inhibited the phosphorylation of the $\mu$2 peptide by AAK1 with an IC$_{50}$ value of 2.8 ± 0.1 nM $(n=3)$, (Fig. 1A). In a panel of 43 functional or binding assays for receptors, transporters and enzymes, BMT-046091 was inactive at up to 5 $\mu$M in all assays (data not included). The high potency and selectivity at AAK1 plus moderate lipophilicity (clog P = 2.4) render BMS-046091 as an excellent candidate to be tritiated as an AAK1 inhibitor radioligand.

$[^{3}H]$BMT-046091 (structure illustrated in Fig. 1A insert) was evaluated in an in vitro binding autoradiographic assay. As shown in Fig. 1B, $[^{3}H]$BMT-046091 exhibited a specific binding pattern in the brain region of the AAK1 wild-type mouse, and the pattern was absent in the AAK1 knockout mouse. The binding pattern was also eliminated by the presence of a high concentration (10 $\mu$M) of BMS-901715, an unlabeled selective AAK1 inhibitor, in rat (Fig. 2A), and cynomolgus monkey sections (Fig. 3B inset). The data suggest that $[^{3}H]$BMT-046091 binds specifically to the AAK1 sites in the brain of rodent and the non-human primate.

Quantitative binding autoradiography was performed to measure the density ($B_{\text{max}}$) and affinity ($K_{d}$) of specific $[^{3}H]$BMT-046091 binding in various brain regions and spinal cord of rats. Fig. 2A shows representative autoradiographic images of $[^{3}H]$BMT-046091 binding generated in sequential rat coronal sections incubated in incremental concentrations of the radioligand from 0.3 to 10 nM in the absence (total binding) and presence (non-specific binding, NS) of 10 $\mu$M BMS-901715. The autoradiogram intensity at each radioligand concentration was measured, and a saturation binding curve and Scatchard plot were then generated to yield $B_{\text{max}}$ and $K_{d}$ for each region, as exemplified in Fig. 2B for the prefrontal cortex and spinal cord dorsal horn. Table 1 summarizes $B_{\text{max}}$ and $K_{d}$ values for all regions examined.

3.2. Distribution of $[^{3}H]$BMT-046091 binding sites in the rat and monkey brain

Fig. 3A and B shows the distribution pattern of $[^{3}H]$BMT-046091 binding in the rat and monkey brain at various coronal levels (hemi-sections). In the rat, high density binding was apparent in all cortical areas including the prefrontal, frontoparietal, cingulate, temporal and occipital cortex, hippocampus and several subcortical areas including the amygdala, septum and accumbens nucleus. A medium level of binding was seen in the olfactory nucleus, stria tum, thalamus, hypothalamus and cerebellar cortex. Overall the brainstem expressed a low level of binding, but the density varied in subregions. The binding in the white matter and fiber bundles such as the corpus colosum was close to the non-specific. The projected maximal density values ($B_{\text{max}}$) of the specific binding in selected brain regions are listed in Table 1. Noticeably, the $B_{\text{max}}$ of the specific $[^{3}H]$BMT-046091 binding in the forebrain regions and the cerebellar cortex was 2–4 fold higher than that in the brainstem and spinal cord dorsal horn. The binding affinity ($K_{d}$) of $[^{3}H]$BMT-046091 measured in the spinal cord dorsal horn and the
brainstem (2–4 nM) appeared higher than that in the forebrain regions (6–12 nM) (Table 1).

It is apparent that the distribution of [3H]BMT-046091 binding in some brain regions was heterogeneous. For example, in the frontoparietal cortex, specific binding tended to be higher in the superficial layers than the deep layers (Fig. 3A). In the hippocampus, [3H]BMT-046091 binding was more abundant in the CA1-CA3 than in the dentate gyrus. In the striatum, [3H]BMT-046091 binding density appeared greater in the caudate putamen than in the globus pallidus. In the thalamus, [3H]BMT-046091 binding was concentrated more in the medial and central than the lateral regions. In the amygdala, [3H]BMT-046091 binding was concentrated in the lateral and basolateral nuclei. In the brainstem, [3H]BMT-046091 binding tended to distribute in the regions containing nuclei. For example, [3H]BMT-046091 binding appeared densely in the periaqueductal gray and ventral tegmental area/substantia nigra of the midbrain, and in the pontine nucleus and the parabrachial nucleus/locus coeruleus region of the pons.
The distribution pattern of \(^{3}H\)BMT-046091 binding in cynomolgus monkey brains was similar to that in the rat (Fig. 3B). A high density of \(^{3}H\)BMT-046091 binding was observed in all cortical areas, amygdala and hippocampal CA1-CA3. A moderate density was present in the striatum, thalamus, hypothalamus and cerebellar cortex. The brainstem expresses a low level of binding sites. Again, like in the rats, \(^{3}H\)BMT-046091 binding distributed heterogeneously in some brain regions of the cynomolgus monkey, e.g., enriching in the superficial layers of the cortex, caudate putamen (vs. globus pallidus), hippocampal CA1-CA3 (vs. dentate gyrus), the brainstem periaqueductal gray and nuclei.

3.3. Distribution of \(^{3}H\)BMT-046091 binding sites in the rat spinal cord

\(^{3}H\)BMT-046091 binding was present in all spinal cord segments, e.g., cervical, thoracic, lumbar and sacral with a distribution similar pattern. Fig. 3C left panel and Fig. 3D show examples of \(^{3}H\)BMT-046091 binding in the spinal lumbar cord (L3-L5) at the level where the sciatic nerve afferents terminate. It appeared that \(^{3}H\)BMT-046091 binding was exclusively located in the gray matter. In the gray matter, the binding sites were predominantly localized in the superficial dorsal horn layers (specifically laminae II and III), and in the central zone (lamina X). The binding density in the spinal cord was relatively low; the \(B_{\text{max}}\) measured in the superficial layers of the spinal dorsal horn was about 2–4 fold less than that in the forebrain areas (Table 1). The binding affinity \(K_{d}\) appeared higher in the spinal dorsal horn (2 nM) than the forebrain regions (6–12 nM).

Next we examined whether \(^{3}H\)BMT-046091 binding density was affected in the superficial dorsal horn of the lumbar segment, in CCI rats 2–3 weeks post-surgery. As shown in Fig. 3C left panel and Fig. 4A, there was no difference of the binding density in the superficial dorsal horn ipsilateral versus contralateral to the sciatic nerve injury. In contrast, when adjacent spinal sections were processed for in vitro binding autoradiography of \(^{3}H\)gabapentin, a voltage gated calcium channel \(\alpha_{2}d-1\) subunit ligand, and \(^{3}H\)PK11195, a microglia activation marker, a significant increase in binding for both radioligands was observed in the superficial dorsal horn ipsilateral to the nerve injury in comparison to the contralateral, as shown in Fig. 3C middle and right panels and Fig. 4A. In addition, we compared \(^{3}H\)BMT-046091 binding in the ipsilateral superficial spinal dorsal horn in CCI versus sham-operated rats. As shown in Fig. 4B, \(^{3}H\)BMT-046091 binding was not altered in CCI rats compared to sham-operated. In contrast, \(^{3}H\)PK11195 was significantly increased in CCI rats.

3.4. Binding site occupancy of AA1 inhibitors measured by ex vivo \(^{3}H\)BMT-046091 binding autoradiography

In the previous study, we have demonstrated that systemically administered AA1 inhibitors, such as LP-935509, produce antinociceptive effects in several rodent models of neuropathic pain.
and suppress hyperactivity of spinal dorsal neurons in CCI rats (Kostich et al., 2016). In this study, we developed an ex vivo [3H]BMT-046091 binding autoradiographic assay to measure binding site occupancy of LP-935509 to understand the relationship of the AAK1 target engagement with the antineuropathic pain effects. As described previously (Kapur et al., 2001; Lengyel et al., 2008; Li et al., 2016), the incubation time and temperature have a profound impact on ex vivo occupancy measurement. Thus, initial studies were conducted to identify optimal ex vivo binding conditions for AAK1 binding site occupancy measurements. As shown in Fig. 4B, binding site occupancy values measured in the parietal cortex from rats dosed with LP-935509 at 10 mg/kg po remained essentially unchanged over the 2–30 min incubation time at the conditions of incubation with 10 nM [3H]BMT-046091 at 22 °C. Using these conditions, we evaluated the dose-dependent occupancy responses of LP-935509 in the spinal cord and parietal cortex (Figs. 3D, 4C and 5B,C). Fig. 3D illustrates an example of representative binding autoradiography images. [3H]BMT-046091 binding density was dose-dependently decreased in the spinal cord of rats dosed with LP-935509 at 10 mg/kg po, indicating that LP-935509 occupied spinal AAK1 binding sites in a dose-dependent manner. Fig. 4C shows the quantitative ex vivo occupancy data on LP-935509 generated from the spinal cord dorsal horn and the parietal cortex. The occupancy values appeared similar between the spinal cord and the parietal cortex.

3.5. Correlation of AAK1 binding site occupancy with antineuropathic pain effects

We further examined the relationship of the AAK1 binding site occupancy with the antineuropathic effect of LP-935509. In this study, following oral dose of LP-935509, rats were evaluated by monitoring thermal hyperalgesia in the CCI rats for 180 min post dose. Immediately after the behavioral test brain tissues were processed for ex vivo binding autoradiography to measure the binding site occupancy. We chose the 180 min time point for assessing the binding site occupancy as the time point showed the maximum behavioral response. As shown in Fig. 5A, LP-935509 produced a dose-and time-dependent reversal of thermal hyperalgesia in the CCI rats. Fig. 5B shows that the mean paw withdrawal latency values are increased with increasing occupancy levels. Fig. 5C shows that the percent reversal of thermal hyperalgesia by LP-935509 correlated well with the AAK1 binding site occupancy of the AAK1 inhibitor.

4. Discussion

In the current study we characterized a potent and selective AAK1 inhibitor radioligand, [3H]BMT-046091, and used it to map the distribution pattern of AAK1 binding sites in the rodent and non-human primate. We found that in both species AAK1 binding sites were broadly expressed in the brain and spinal cord with the highest level in the forebrain. In the spinal cord, AAK1 binding was enriched in the superficial layers of the dorsal horn. The AAK1 binding the spinal dorsal horn was not altered after CCI surgery. Furthermore, we developed an ex vivo [3H]BMT-046091 binding autoradiographic assay to determine the target engagement of AAK1 inhibitors. We found that orally dosed LP-935509 occupied AAK1 binding sites in a dose-dependent matter and the target occupancy was correlated with the antineuropathic pain effect of LP-935509.

BMT-046091 exhibited an IC50 of ~3 nM in inhibiting the AP2 μ2...
peptide phosphorylation and >5 μM potency for 43 off-targets including receptors, transporters and enzymes. [3H]BMT-046091 binding in the brain was absent in the AAK1 KO mouse, and could be completely displaced with a high concentration of a non-radiolabeled AAK1 inhibitor in the rat and non-human primate, indicating that [3H]BMT-046091 binds to the specific AAK1 sites. The distribution pattern of AAK1 binding was comparable in the rat and cynomolgus monkey with widespread expression throughout the brain and spinal cord. AAK1 binding was present only in the gray matter, indicating their association with the neuronal cell bodies and terminals. AAK1 mRNA expression has been mapped in the mouse brain, and the distribution appears to be relatively uniform (Allen Brain Atlas, Allen Institute). The heterogeneity of AAK1 protein binding pattern in the brain observed in the present study may indicate selective association of AAK1 with certain neuronal systems and potentially specific roles in regulation of neuronal functions in the CNS.

Inhibition of AAK1 in the spinal cord appears to be sufficient for mediating the antineuropathic pain effect of AAK1 inhibitors (Kostich et al., 2016). However, the involvement of supraspinal AAK1 in neuropathic pain and the antineuropathic pain effect of AAK1 inhibition cannot be ruled out. Indeed, many of the brain regions expressing AAK1 are part of the brain network for pain and its modulation. For example, the prefrontal and frontoparietal cortical areas are involved in pain signal integration and cognitive modulation, whereas the cingulate cortex and amygdala play an important role in regulating the affective and emotional component of physiological and pathological pain. On the other hand, the brainstem periaqueductal gray and parabrachial nucleus-locus coeruleus provide the descending pathway modulation of pain. Chronic and neuropathic pain promotes neuroplastic changes in these supraspinal brain regions as well as the spinal dorsal horn as the anatomical substrates for central sensitization (Latremoliere and Woolf, 2009). Thus, the widespread AAK1 expression in these brain regions may be the additional target for AAK1 inhibitors to produce the antineuropathic pain action.

In this study, we demonstrated the presence of AAK1 binding sites spreading throughout the spinal gray matter across all levels of the spinal cord. The highest expression appears in the dorsal horn superficial layers, where primary nociceptive C-fibers terminate (lamina II) and projection sensory neurons are located (lamina III). The enriched expression of AAK1 in the spinal dorsal horn, a relay station in pain transmission, provides an anatomic substrate for the involvement in neuropathic pain and for mediating the antineuropathic pain action of AAK1 inhibitors (see below for further discussion). The relative contribution to AAK1 binding sites originated from AAK1 expressing in superficial dorsal horn cells vs. from the dorsal root ganglion neurons is currently unknown, but would be worth of exploration.

AAK1 expression was not altered in the lumbar cord dorsal horn after CCI surgery. In contrast, [3H]PK11195 and [3H]Gabapentin binding was significantly increased. [3H]PK11195, a mitochondrial benzodiazepine receptor ligand, is a biomarker of microglia activation and neuroinflammation. Upregulation of [3H]PK11195 binding in the dorsal horn has been reported in inflammatory and neuropathic pain (Miller et al., 2013; Imamoto et al., 2013). Gabapentin, a ligand to the voltage-gated calcium channel (VGCC) 2δ-1 subunit, is effective in treatment of neuropathic pain. The VGCC 2δ-1 protein is upregulated in the dorsal root ganglion and the dorsal horn in the spinal nerve ligation model of neuropathic pain (Nieto-Rostro et al., 2014). Upregulation of both markers has been linked to neuroplastic changes in the dorsal horn responsible for central sensitization and neuropathic pain development (Miller et al., 2013; Imamoto et al., 2013; Patel et al., 2013). AAK1 at least at the level of expression does not appear to be part of the neuroplastic changes occurring after peripheral nerve injury. However, it is possible that the biophysical property of AAK1 can be altered in neuropathic pain, which in turn alters AP2 functionality and, therefore, clathrin-mediated receptor internalization, a part of mechanisms thought to underlie neuropathic pain development and maintenance (Chen et al., 2013).

In the previous study, we have demonstrated that AAK1 knockout mice exhibited an antinoceptive phenotype in the formalin phase II model of chronic pain and the spinal nerve ligation (SNL) model of neuropathic pain (Kostich et al., 2016), and
AAK1 inhibition by small molecule AAK1 inhibitors recapitulates the AAK1 knockout antinociceptive phenotype. We have found that the antineuropathic pain responses of AAK1 inhibitors required distribution of AAK1 inhibitors to the CNS following systemic administration. For example, the highly brain penetrant LP-935509 was effective in reversing nociceptive responses in a variety of models of chronic and neuropathic pain in mice and rats. In contrast, a compound with poor brain penetration was not antinociceptive (Kostich et al., 2016). Furthermore, inhibition of AAK1 activity by intrathecal delivery of an AAK1 inhibitor into the spinal cord attenuated nociceptive responses inCCI rats. Likewise, microi ontophoretic application of an AAK1 inhibitor into the dorsal horn blocked nociceptive fiber activation-induced windup in nociceptive dorsal horn neurons. While these data indicate the essential role of AAK1 inhibition in the spinal cord in antinociceptive effects of AAK1 inhibitors, they do not provide direct evidence of the engagement of AAK1 inhibitors to the AAK1 target in the spinal cord. Thus, in this study we tested whether the target occupancy of AAK1 inhibitors could be measured using an ex vivo \([^{[1]}H]BMT-046091 binding autoradiographic assay. We found that the ex vivo \([^{[1]}H]BMT-046091 binding exhibited a sufficient signal/noise window with no detectable dissociation of tested AAK1 inhibitors during the ex vivo binding process. Using the assay, we first evaluated AAK1 binding site occupancy of orally administered LP-935509 in naive rats. We found that LP-935509 dose-dependently occupied AAK1 binding sites in the spinal cord dorsal horn, and the occupancy values measured in the spinal cord appeared to be in line with those measured in the forebrain parietal cortex, suggesting no potency difference of LP-935509 in occupying AAK1 in the spinal cord vs. the forebrain areas. Further testing of tissue samples of CCI rats collected following LP-935509 dosing and thermal hyperalgesia testing showed a correlation of the binding site occupancy values of LP-935509 with the antineuropathic pain effects.

In summary, AAK1 binding sites are broadly expressed in the brain and spinal cord. In the spinal cord, AAK1 binding is enriched in the superficial dorsal horn and the binding density is not changed following chronic sciatic nerve constriction injury. A systemically administered AAK1 inhibitor occupies AAK1 binding sites and the AAK1 binding site occupancy is correlated to the antineuropathic pain effect. \([^{[1]}H]BMT-046091 binding autoradiography can be used to understand the neurobiology of AAK1 protein and to determine the pharmacokinetic-pharmacodynamic relationship of AAK1 inhibitors.

References


