Chemical genetic identification of GAK substrates reveals its role in regulating Na⁺/K⁺-ATPase

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Cyclin G–associated kinase (GAK) is a ubiquitous serine/threonine kinase that facilitates clathrin uncoating during vesicle trafficking. GAK phosphorylates a coat adaptor component, AP2M1, to help achieve this function. GAK is also implicated in Parkinson’s disease through genome-wide association studies. However, GAK’s role in mammalian neurons remains unclear, and insight may come from identification of further substrates. Employing a chemical genetics method, we show here that the sodium potassium pump (Na⁺/K⁺-ATPase) α-subunit Atp1a3 is a GAK target and that GAK regulates Na⁺/K⁺-ATPase trafficking to the plasma membrane. Whole-cell patch clamp recordings from CA1 pyramidal neurons in GAK conditional knockout mice show a larger change in resting membrane potential when exposed to the Na⁺/K⁺-ATPase blocker ouabain, indicating compromised Na⁺/K⁺ change in resting membrane potential when exposed to the Na⁺/K⁺-ATPase blocker ouabain, indicating compromised Na⁺/K⁺-ATPase function in GAK knockouts. Our results suggest a modulatory role for GAK via phosphoregulation of substrates such as Atp1a3 during cargo trafficking.

DOI 10.26508/lsa.201800118 | Received 26 June 2018 | Revised 7 December 2018 | Accepted 10 December 2018 | Published online 31 December 2018

Introduction

Cyclin G–associated kinase (GAK), also known as auxilin 2, is a member of the Numb-associated kinase (NAK) family. Other members include adaptor-associated kinase 1 (AAK1), BMP2-inducible kinase (BIKE/BMP2), and myristoylated and palmitoylated serine/threonine kinase 1 (MPSK1) (Smythe & Ayscough, 2003; Sorrell et al., 2016). GAK is ubiquitously expressed in various tissues (Kanaoka et al., 1997; Kimura et al., 1997) and is primarily known for its role in clathrin uncoating (Zhang, Engqvist-Goldstein et al., 2005b; Lee et al., 2005; Lee et al., 2006). At its N-terminus, GAK has a highly conserved serine/threonine kinase domain followed by a lipid-binding PTEN-like domain (Lee et al., 2006), a clathrin-binding domain (Kametaka et al., 2007), and a J-domain, the latter two domains being sufficient for Hsc70 and GAK-mediated uncoating of clathrin-coated vesicles (Greener et al., 2000; Umeda et al., 2000). Conventional GAK knockout in mice is embryonic lethal, while conditional knockout of GAK in the developing brain, liver, and skin all result in death shortly after birth (Lee et al., 2008). Knockout of GAK in adult mice using a tamoxifen-inducible Cre recombinase also resulted in death (Lee et al., 2008). A transgenic mouse expressing only the clathrin-binding and J-domains was able to rescue the lethality of GAK knockout in the liver and brain, showing that neither the PTEN-like domains nor the kinase domain was necessary for GAK function in these tissues (Park et al., 2015). However, kinase-dead GAK knock-in mice still led to neonatal lethality caused by defective lung development (Tabara et al., 2011), indicating importance for GAK activity. As a serine/threonine kinase, GAK exerts part of its functional effects by targeting downstream substrates. The only currently known substrates of GAK are the μ2- and μ1-subunits of the adaptor proteins AP-2 and AP-1, respectively (Umeda et al., 2000; Korichuk & Banting, 2002; Kametaka et al., 2007), and protein phosphatase 2A (Naito et al., 2012). However, the functional role of GAK is still not fully understood in neurons, and a number of unidentified GAK substrates likely remain.

The sodium potassium pump (Na⁺/K⁺-ATPase, NKA) is a transmembrane ion pump critical in the maintenance of resting membrane potential (RMP). It utilizes the hydrolysis of an ATP molecule to drive the extrusion of three Na⁺ ions from the cell and uptake of two K⁺ ions into the cell against their electrochemical gradients. Structurally, it is a heteromer consisting of three sub-units: α, β, and γ. In mammals, there are four catalytic α-subunits, α1–4. The α1–3-subunits are expressed in brain tissue at varying levels, with α1 being ubiquitous, α2 mainly glial, and α3 neuronal (Bottger et al., 2011). There are three β-subunits: the β1-subunit is expressed ubiquitously, the β2 isoform is expressed in brain and muscle (Blanco, 2005), and the β3 isoform is mainly expressed in lung, testis, skeletal muscle, and liver (Malik et al., 1996; Arystarkhova & Sweadner, 1997). In some tissues, there is also an additional modulatory γ subunit, the FXYD protein, of which there are seven (Sweadner & Rael, 2000; Crambert & Geering, 2003). The NKA undergoes a number of posttranslational modifications that...
affect its trafficking and function, including ubiquitination (Coppi & Guidotti, 1997; Thewenod & Friedmann, 1999) and phosphorylation (Poulsen et al, 2010). Mutations in ATPIA3 are known to cause rapid-onset dystonia parkinsonism (de Carvalho Aguiar, Sweadner et al, 2004; Bottger et al, 2011), as well as alternating hemiplegia of childhood and CAPOS (cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss) syndrome (Clausen et al, 2017).

Identification of direct kinase substrates can reveal previously unknown molecular and cellular kinase functions, which is critical for disease-relevant pathway expansion where kinase–disease genetic links are well established. Direct kinase substrate identification is a challenging problem in cell signalling. In this study, we engineered the kinase domain of GAK to enable its use of bulky ATP analogs with γ-thiophosphates. Analog-specific GAK was then used to label its substrates specifically. These substrates and their phosphorylation sites were identified with mass spectrometry, revealing putative novel GAK substrates. Using phosphospecific antibodies, we validated Atp1a3, the catalytic α3-subunit of NKA, and Sipa1l1 (signal-induced proliferation-associated 1-like protein 1) as novel GAK substrates. Using overexpression studies in neurons, COS7, and human embryonic kidney (HEK) 293T cells, we show that Atp1a3 T705 is phosphorylated by GAK and another NAK family kinase AAK1, and it is important for NKA localization and function. In order to further study GAK function in neurons, we crossed GAK-floxed (F) mice with neuronal Cre lines to obtain conditional GAK knockout mouse models. We show that GAK knockout neurons were more sensitive to NKA inhibition by ouabain, indicating alterations in NKA function. Our results describe a novel role for GAK in regulating ATP1a3 function.

Results

GAK protein engineering and site suppressor rescue mutations

To identify downstream targets of GAK in brain tissue, we used a chemical genetic method of labelling kinase substrates (Blethrow et al, 2004; Hertz et al, 2010). To do so, we first had to engineer a pocket in the GAK protein. Using a truncated form of GAK from aa 1–400 (GAK1–400) containing the GAK domain (aa 54–311), we generated two ATP analog–specific (AS) mutants, T123G and T123A, by mutating the hydrophobic “gatekeeper” residue of the GAK ATP-binding pocket to a smaller aa (Fig 1A). This enlarges the ATP-binding pocket and allows ATP analog specific mutants to utilize bulky ATP-γ-S analogs containing a thiol group to selectively thiophosphorylate their substrates (Fig 1B). Thiophosphorylated substrates can be detected via Western blot after alkylation by p-nitrobenzylmesylate (PNBM) using an anti-thiophosphate ester antibody (Allen et al, 2007). To determine which bulky ATP-γ-S analog was most compatible with GAK mutant kinases, we performed an in vitro kinase reaction using GAK autophosphorylation as a readout of GAK activity. We found that GAK T123A could use furfuryl-ATP-γ-S, but at reduced levels (Fig 1C), showing GAK to be an intolerant kinase. To rescue this loss, we needed to identify potential secondary-site suppressor mutations. We mutated the αa residue preceding the conserved “DFG” motif, C190, to alanine or threonine, as the site was previously reported to rescue the activity of intolerant kinase NDR1 (Ultanir et al, 2012). We also introduced mutations at a second aa site that comes into close contact with the N-6 position of ATP adenine (Ehlen et al, 2003), V99 in GAK. However, we observed no changes in GAK activity with any of these strategies (Fig S1). We next took a closer look at the structure of GAK in comparison with other kinases. A key component known to affect kinase activity is the regulatory spine, which consists of four hydrophobic residues spanning the tertiary structure of the kinase domain (Kornev et al, 2006). The introduction of isoleucine, a hydrophobic β-branched aa, at a –2 position with respect to the gatekeeper residue located on the β5 strand (Fig 1D), is able to compensate for the loss of hydrophobicity caused by gatekeeper mutation (Joseph & Andreotti, 2011). In GAK, the hydrophobic spine residues are L89, F101, H171, and F192 (Fig 1D). We therefore introduced a L121I mutation to the GAK T123A mutant and observed that L121I moderately rescued kinase activity (Fig 1E). Finally, we made use of GAK structure–based sequence alignment with the other NAK family members (Zhang, Kenski et al, 2005a) using mouse protein sequences and noticed that the first hydrophobic spine residue L89 was a methionine in all other members, as well as in the human GAK sequence. To mimic AAK1’s sequence in that region, we introduced a C87Q/F88I/L89M mutation to the L121I/T123A GAK mutant (Fig 1A and D). We found that these rescue mutations together (AS) restored GAK activity towards furfuryl-ATP-γ-S to levels comparable with GAK WT using ATP-γ-S (Fig 1E). We used this final combination of mutations T123A/L121I/C87Q/F88I/L89M in GAK1–400 (referred to as GAK AS) during subsequent experiments to thiophosphorylate putative substrates in mouse brain tissue.

Chemical genetic identification of GAK substrates

Before performing covalent capture, we first wanted to confirm and characterize the presence of GAK in the wildtype mouse brain. GAK is expressed from postnatal day (P)4–P50 (Fig S2A) throughout all brain regions (Fig S2B).

In order to perform covalent capture for kinase substrate identification, GAK1–400 was first purified from insect cells, as full length (FL) GAK was not stable enough to purify. GAK AS was purified along with a K69R kinase dead (KD) mutant of GAK1–400 (GAK KD) as a negative control. P11 mouse brain lysates were then incubated with furfuryl-ATP-γ-S and either purified GAK KD or GAK AS to label potential substrates. Afterwards, brain lysate proteins were digested with trypsin. Any thiol-containing peptides (Fig 2A, red), including those with cysteine aas (Fig 2A, blue), were captured with iodoacetyl agarose beads, while peptides phosphorylated by endogenous kinases (Fig 2A, grey) were washed out. Using oxone-induced hydrolysis, cysteine-linked peptides were left on the beads while thiophosphate ester–linked peptides were released as phosphopeptides (Fig 2A, red). These eluted peptides were then subjected to liquid chromatography/tandem mass spectrometry analysis to identify substrates and their phosphorylation site (Fig 2A).

From the data generated, only phosphopeptides detected at least three times in GAK AS samples and not in any GAK KD negative controls are included in a list of substrate candidates. Of the
potential GAK substrates identified, there appears to be a predominant preference for a small TG motif, where T is phosphorylated threonine and G is glycine (Fig 2A). This motif matches with that of a previously identified GAK and AAK1 substrate, the T156 residue of the AP-2 adaptor complex μ2-subunit (SQITSQVTG-QIGWRR) (Korolchuk & Banting, 2002; Ricotta et al, 2002; Zhang et al, 2005b), as well as the GAK substrate protein phosphatase 2A (PP2A) B’γ T104 residue (SNP-TGAEFPD) (Naito et al, 2012). Therefore, in the list of putative GAK substrates, we removed the three candidates that do not contain the “TG” motif (Table 1). The dataset was uploaded in PRIDE # PXD011319. Neither AP2 μ2 nor PP2A B’γ appeared in our list of candidate substrates, which could be due to either low abundance or that using GAK1–400 instead of FL GAK resulted in less effective interaction with binding partners. In addition, due to their biophysical properties, some peptides may not be detected by mass spectrometry. Therefore, not detecting a...
phosphorylation site would not necessarily mean that it is not phosphorylated. The putative substrates themselves appear to reflect the ubiquitous nature of GAK (Table 1). We chose to further study two of these substrates due to their important functions in neurons: Sipa1L1, which regulates dendritic spine morphology, and the catalytic α-subunits of the sodium potassium pump

Table 1. Table of potential GAK substrates.

| Position | Leading proteins | Protein names | Gene names | Sequence window |
|----------|------------------|---------------|------------|-----------------|
| T425     | Q9QW16           | SRC kinase signalling inhibitor 1 | Srcin1 | GSPVHHAERLGGAAPTGGVGSPSPSAILERR |
| T55      | Q9Q6F9           | Tubulin β-4A chain | Tubb4a | SDLQLERINYYNEATGGNYPRAVLVDLEP |
| T472     | Q88532           | Zinc finger RNA-binding protein | Zfr | NTSSIATSSVGLSTGNSSLNSTNKTSA |
| T249     | Q8C0T5           | Signal-induced proliferation-associated 1-like protein 1 | Sipa1l1 | SDRGPTKLSDFLTGGGKGSGFLSDLVIDG |
| T385     | Q3UIZ0           | Cyclin-GAK | Gak | GGFDLIRGLGTERLTNLKDTSKIVOSVAN |
| T705     | Q8VEC0           | Sodium/potassium-transporting ATPase subunit α-3; α-1; α-2 | Atp1a3;Atp1a1;Atp1a2 | IIIVEGQRGAIAYVTDGGVNDSPALKKADI |
| T3       | Q64213           | Splicing factor 1 | Sf1 | __________MATGAATPLDPFSPKRK |
| T55      | P68372           | Tubulin β-4B chain | Tubb4b;Tubb2c | SDLQLERINYYNEATGGNYPRAVLVDLEP |
| T118     | P62002           | Myelin proteolipid protein | Plp1 | DYTICTICGKSATYTGQGKRGSRGQHQA |
| T465     | A2ARP8           | Microtubule-associated protein 1A MAP1 light chain LC2 | Mtap1a;Map1a | MQFLMQWKANSAKTGVILANGKEAESVVP |
| T627     | Q3UR70           | Transforming growth factor-β receptor-associated protein 1 | Tgfrap1 | LAILYLEELRQVSTGGKDEVATETQAKLK |

GAK regulates Na+/K+-ATPase Lin et al. https://doi.org/10.26508/lsa.201800118 vol 1 | no 6 | e201800118 4 of 20
GAK phosphorylates Sipa1L1 at T249 in HEK 293T cells

Sipa1L1, also known as SPAR, was first identified as a GTPase-activating protein for Rap that was targeted to dendritic spines, where it induced reorganization of F-actin filaments to promote dendritic spine head growth (Pak et al., 2001). It is a large, multi-domain protein containing two separate regions (Act1 and Act2) capable of independently reorganizing F-actin (Fig S3A) (Pak et al., 2001). From our GAK substrate screen, we identified the T249 site, located in the Sipa1L1 Act1 domain (aa 1–627) as the site of GAK phosphorylation (Fig S3A). The T249 site is conserved between mouse, rat, and human Sipa1L1, but is not conserved between Sipa1L1 and its isoforms (Sipa1L2 and Sipa1L3). In order to study Sipa1L1 phosphorylation, we generated an Act1 domain–only form of Sipa1L1 (Act1) (Fig S3A). Firstly, we tested whether GAK and Sipa1L1 are able to interact by co-expressing myc-tagged GAK FL with either HA-tagged Sipa1L1 or HA-tagged Act1 in HEK 293T cells and immunoprecipitating GAK. We find that GAK and Sipa1L1 do interact and that Act1 can coimmunoprecipitate with GAK (Fig S3B). To test whether or not GAK phosphorylates Sipa1L1 at T249, we generated a T249A mutant (TA). We then overexpressed GAK FL WT or GAK FL KD with either Act1 WT or TA in HEK 293T cells. Our results show that GAK is the primary kinase phosphorylating Atp1a3 at T705.

GAK phosphorylates Atp1a3 at novel site T705 in HEK 293Ts

(A) Atp1a3 is immunoprecipitated by GAK from HEK 293T cells co-transfected with myc-tagged FL GAK WT and HA-tagged Atp1a3 WT (left panel). Atp1a3 is also able to be immunoprecipitated by myc when cells are co-transfected with myc-tagged GAK1-400 and HA-tagged Atp1a3 WT (right panel). HA-tagged and myc-tagged pRK5 vectors were used as IP controls. (B) Atp1a3 pT705 is present in HEK 293T samples co-transfected with Atp1a3 WT and either FL GAK WT or GAK1-400 (left). Atp1a3 T705A mutants show reduced pT705, as do GAK KD mutants (centre). GAK1-400 pT705 levels are similar compared to FL GAK (right). Graph data represent means ± SEM, n = 3 independent experiments. Graph FL WT/T705 P < 0.001, KD/T705 P = 0.001, KD/T705/WT = 0.009. GAK1-400 WT/T705 P = 0.004, KD/T705/WT = 0.005, KD/T705/WT P = 0.09. (C) HEK 293T cells co-transfected with HA GAK FL WT and HA-Atp1a3 WT and pretreated in serum-free media were incubated with either DMSO or 5 μM of GAK inhibitor 12g, 12i, or 12g+i for 1 h n = 3 independent in duplo experiments. Graph data represent means ± SEM, 12g P = 0.03, 12i P = 0.04, 12g+i P = 0.009; 12g P = 0.009, 12i P = 0.009, 12g+i P = 0.03; *P < 0.05, **P < 0.01, ***P < 0.001, t test. (D) Co-transfections of HEK 293T cells with HA-tagged Atp1a3 WT and either HA-tagged GAK or AAK1 demonstrate that GAK is the primary kinase phosphorylating Atp1a3 at T705. n = 5 independent experiments.
we co-expressed myc-tagged FL GAK with HA-tagged Atp1a3 in HEK 293T cells and immunoprecipitated GAK to confirm pull down of Atp1a3 (Fig 3A, left panel). We also tested to see whether myc-tagged GAK^{1–400} was able to co-immunoprecipitate HA-tagged Atp1a3. Here, we immunoprecipitated with myc instead of GAK due to the truncation of the C-terminus and found that the GAK domain is able to pull down Atp1a3 (Fig 3A, right panel).

To validate Atp1a3 T705 as a GAK phosphorylation site, we generated a rabbit polyclonal phosphospecific antibody against Atp1a3 T705 (pT705). We made an Atp1a3 phosphomutant, Atp1a3 T705A (TA), which cannot be phosphorylated, and then overexpressed FL GAK wildtype (GAK FL WT) or KD (GAK FL KD) with either Atp1a3 wildtype (WT) or Atp1a3 TA in HEK 293T cells. To determine whether the kinase domain alone is sufficient to phosphorylate Atp1a3 T705, we also overexpressed GAK^{1–400} WT or KD (GAK^{1–400} KD) with either Atp1a3 WT or Atp1a3 TA. Our results suggest that Atp1a3 T705 is phosphorylated by GAK and that the GAK domain is as sufficient as FL GAK (WT/WT HA-GAK^{1–400} 92.8 ± 21.3; Fig 3B, right). We observed a large reduction in pT705 levels in cells co-transfected with the KD form of GAK (KD/WT 22.7 ± 9.6; Fig 3B, centre) and GAK^{1–400} (KD/WT 42.9 ± 20.4; Fig 3B, centre) when normalized to their respective WT/WT controls. This reduction was also seen with the Atp1a3 TA mutant for both GAK FL (WT/TA 46.2 ± 14.4; KD/TA 25 ± 13.5; Fig 3B, centre) and GAK^{1–400} (WT/TA 48.3 ± 16.5; KD/TA 36.3 ± 13.5; Fig 3B, centre). To further confirm that GAK activity causes Atp1a3 T705 phosphorylation, we treated HEK 293T cells co-transfected with GAK FL WT and Atp1a3 WT with GAK inhibitors 12g IVAP-1966 and/or GAK inhibitor 12i IVAP-1967 (5 μM each) for 1 h under serum-free conditions (Kovackova, Chang et al, 2015a). Both GAK inhibitors reduced Atp1a3 T705 phosphorylation (12g 57.5 ± 12.9, 12i 63.3 ± 7.47, 12g+i 69.1 ± 9.26) compared to DMSO (100 ± 7.73) (Fig 3C). However, these GAK inhibitors do not fully eliminate phosphorylation of T705. It is a possibility that other members of the NAK family might also target Atp1a3 T705. For example, both AAK1 and GAK phosphorylate AP-2 μ (Korolchuk & Banting, 2002; Lee et al, 2005; Ricotta et al, 2002). To examine whether AAK1 is able to phosphorylate Atp1a3 at T705, we co-transfected Atp1a3 WT or Atp1a3 TA with either AAK1 WT or AAK1 KD and then ran an immunoblot to examine Atp1a3 pT705 levels. We find that while AAK1 WT can phosphorylate Atp1a3 WT, the level of phosphorylation by AAK1 WT is much weaker compared to GAK WT (Fig 3D). These experiments show that GAK, and possibly other NAK family members, can phosphorylate Atp1a3 at T705 in cells. Interestingly, mutations in the ATP1A3 gene are known to cause rapid-onset dystonia parkinsonism (de Carvalho Aguiar et al, 2004). Considering the critical role of Atp1a3 in neuronal function, we decided to investigate Atp1a3 further.

Endogenous phosphorylation of Atp1a3 is regulated by phosphatases.

We next examined endogenous pT705 levels in neural tissue. Endogenous pT705 is readily detectable in embryonic day (E18) rat brains (E18 pT705 101 ± 2.3; Fig 4A) despite lower expression of total Atp1a3 in rat embryos compared to adult rat (Orlowski & Lingrel, 1988) (E18 Atp1a3 6.7 ± 2.7; adult Atp1a3 99.8 ± 1.9; Fig 4A). However, the amount of pT705 in adult rats (adult pT705 22.6 ± 3.1; Fig 4A) appears lower despite increased abundance of total Atp1a3 (Fig 4A), which might suggest a potential developmental role for Atp1a3 T705 phosphorylation. GAK protein levels are detectable in fairly similar amounts in adult rat brain compared to embryonic brain (E18 GAK 109.7 ± 4.4; adult GAK 116 ± 4.0; Fig 4A).

To test the phosphatase-dependent regulation of pT705, we treated rat primary cortical cultures with phosphatase inhibitors okadaic acid (OA), cyclosporin A (CSA) and DMSO vehicle. Phosphorylation levels were increased upon phosphatase inhibitor treatment, most notably OA. To ensure that our antibody is specific, we dephosphorylated either DMSO-, OA- or CSA-treated samples by calf alkaline phosphatase (CIP) treatment of protein lysates. CIP-treated samples clearly demonstrated the loss of pT705 (Fig 4B, lanes 2, 4, 6), while total Atp1a3 levels remained constant. These experiments show that endogenous pT705 phosphorylation is present in neurons and is negatively regulated by phosphatases PP1, PP2A, and PP2B (calcineurin). Our results indicate that Atp1a3 T705 phosphorylation occurs in cultured neurons and in vivo.

Atp1a3 function is important for maintaining RMP after large rapid sodium influxes such as those observed during action potentials.
potential firing (Azarias et al, 2013). Therefore, to investigate whether T705 is regulated by neuronal activity, we treated rat primary cultures for 30 min with different compounds in order to stimulate or block neuronal activity. Neither increase in extracellular potassium (KCl) nor glutamatergic receptor stimulation (glutamate), had an effect on intracellular Na+, as introducing extracellular K+ causes depolarization. Similarly, KCl treatment also results in an initial increase in intracellular Na+, as introducing extracellular K+ causes depolarization. Interestingly, we observed that blocking voltage-gated sodium channels with tetrodotoxin (TTX), which reduces baseline Na+ in nerve cells, increased T705 phosphorylation (TTX 200 ± 17.1), while total levels of Atp1a3 remained unchanged, suggesting that Atp1a3 T705 phosphorylation may be occurring under conditions where intracellular Na+ is reduced.

Figure 5. Atp1a3 phosphomutants show alterations in subcellular localization.
(A) Co-transfections of either YFP-β1 (black) or YFP-β2 (red) with HA-tagged Atp1a3 WT, TA, and TD in HEK 293T cells show different immunoprecipitated glycosylated forms of the ATPase β-subunit. Solid arrows indicate lower MW intracellular forms, and empty arrows indicate the higher MW plasma membrane forms of the β-subunit. Graph data represent means ± SEM, n = 5 independent experiments, TA/β1 intra P < 0.0009, TD/β1 intra P = 6.2 × 10−4, TA/β2 intra P = 2.5 × 10−6, TA/β2 membrane P = 0.05, TD/β2 intra P = 4.4 × 10−12, TD/β2 membrane P = 0.04; **P < 0.05, ***P < 0.01, ****P < 0.001, ****P < 0.0001, t test.
(B) COS7 cells transfected with HA-tagged Atp1a3 WT with DMSO treatment as negative control, dual NAK family inhibitor treatment 12i (VAPI-1967 (12i) + LP-935509 (LP) and Atp1a3 T705A mutant cellular distribution are shown (red is HA immunostaining showing Atp1a3, blue is DAPI). Scale bar = 25 μm.
(C) Atp1a3 staining intensity was normalized to total intensity value for each cell. Y axis shows normalized distance from cell’s centre (1) to its periphery (0). The plots show average and standard error of mean (n = 23, 34, 31 cells for Atp1a3 + DMSO, Atp1a3 + 12i + LP, Atp1a3 T705A, respectively). (D) Data points at the peak for the control group at periphery are compared using ANOVA and paired t tests for individual groups. P-values of these comparisons are P = 0.06 Atp1a3 + 12i, **P < 0.01 Atp1a3 + 12i & LP, ****P < 0.0001 Atp1a3 + 12i, ****P < 0.0001 Atp1a3 T705A + DMSO.

Atp1a3 phosphorylation alterations in trafficking

We next investigated whether phosphorylation at this novel site could play a regulatory role in Na+/K+-ATPase trafficking. It is well known that ER assembly of NKA α- with β-subunits is required for export of the pump to the Golgi apparatus, with the β-subunit acting as a molecular chaperone to facilitate maturation and correct targeting of the pump to the plasma membrane (Geering, 2001, 2008). Prior to NKA insertion at the cell surface, α-subunits must be assembled with β-subunits to form a functioning enzyme. Therefore, we first evaluated whether phosphomutant α3-subunits were able to correctly assemble with β-subunits in order to reach the plasma membrane. Since expression of the β3 isofrom is mainly in the lung, liver, and testis rather than in the brain (Malik et al, 1996; Arystarkhova & Sweadner, 1997), we examined only the ability of β1- and β2-subunits to interact with various Atp1a3 phosphomutant α-subunits. In addition to the Atp1a3 TA mutant, we generated a putative phosphomimetic Atp1a3 T705D (TD) mutant. We co-transfected HA-tagged Atp1a3 WT, TA, or TD phosphomutants with either YFP-β1 or YFP-β2 into HEK 293T cells and then immunoprecipitated α3-subunits with anti-HA antibody conjugated beads. We found that β subunits, detected using a GFP antibody,
precipitated with all of the Atp1a3 phosphomutants, indicating that they are capable of assembly with both YFP-β1 and YFP-β2 (Fig 5A).

However, the Atp1a3 phosphomutants immunoprecipitated different glycosylated forms of the β-subunit (Fig 5A). Structurally, the extracellular domain of the β-subunit has three N-linked glycosylation sites and three disulphide bonds conserved among all isoforms. We observed that there are two main N-glycosylated forms of the β-subunit, with a broader, higher molecular weight band (~80 kDa, ~90 kDa) representing mature subunits with complex-type glycans at the plasma membrane and a lower, sharper band (~65 kDa β1, ~75 kDa β2) representing intracellular immature subunits with only high mannose–type N-glycans in the ER (Tokhtaeva et al, 2010) (Fig 5A). We show that Atp1a3 WT precipitated both glycosylated forms of the β1- and β2-subunits, with a preference for the higher band indicative of mature subunits at the plasma membrane (Fig 5A). In contrast, the Atp1a3 TA phosphomutants did not heavily associate with the higher glycosylated form of the β2-subunit (YFP-β2/TA 16.3 ± 7.12; Fig 5A), and although the TA phosphomutants did associate with both glycosylated forms of the β1-subunit, the higher band showed a large reduction when normalized to WT (YFP-β1/TA 58.3 ± 8.98; Fig 5A), suggesting fewer heterodimers of this assembly at the plasma membrane. There was no change observed in the relative intensity of the lower intracellular band of TA mutants normalized to WT when co-transfected with YFP-β1 (101 ± 3.37), but there was a decrease found when co-transfected with YFP-β2 (82.7 ± 7.63; Fig 5A). The putative phosphomimetic TD only interacted with the lower molecular weight β subunit, indicating ER retention (YFP-β1/TD top band 6.59 ± 1.18; YFP-β2/TD top band 2.81 ± 1.15; YFP-β1/TD bottom band 95.0 ± 3.71; YFP-β2/TD bottom band 80.5 ± 7.97; Fig 5A). To further assess whether TA and TD mutants are able to reach the plasma membrane, surface biotinylation experiments were performed. β-Subunits with complex-type glycans seen at higher molecular weight were highly enriched in the surface fraction (Fig S4A), in agreement with previous studies. However, the lower molecular weight β-subunits also seem to be present at the surface (Fig S4). There was a large reduction in TA mutants at the surface (YFP-β1/TA 44.1 ± 3.9; YFP-β2/TA 57.7 ± 2.7; Fig S4A). As TD is likely sequestered in the ER, the TD bands observed (YFP-β1/TD 15 ± 1.4; YFP-β2/TD 46.1 ± 4.7; Fig S4A) suggest insufficient washing to remove residual biotin prior to lysis, which may indicate a further reduction in TA at the surface than observed. These results indicate that the phosphomimetic TD mutant does not leave the ER and surface expression of the non-phosphorylatable TA mutant is compromised, indicating a role for T705 in trafficking.

Next, we evaluated changes in the distribution of Atp1a3 upon pharmacological inhibition of the NAK kinase family in COS7 cells using light microscopy. Since Atp1a3 can be phosphorylated by AAK1 as well as GAK in cells (Fig 3C), we decided to use a GAK inhibitor 12i IVAP-1967 (Kovackova, Chang et al, 2015b) and a potent inhibitor for NAK family kinases AAK1 and BIKE, LP-935509 (Kostich et al, 2016). For estimating NAK distribution within cells, we used the Fiji plugin, Automated Detection and Analysis of ProTrusions (ADAPT) (Barry et al, 2015). ADAPT maps fluorescence distributions by successively eroding a binary representation of a cell and calculating the mean fluorescence intensity at the periphery of the binary cell at each iteration of the erosion operation. We observed that in COS7 cells that are transfected with Atp1a3 with excess GFP-tagged β subunit, Atp1a3 localizes mostly to the cell periphery (Fig 5B and C). This preferential peripheral distribution is significantly reduced upon simultaneous treatment with NAK family kinase inhibitors 12i (IVAP-1967 (12i) and LP-935509 (LP) (Fig 5B–D). Atp1a3 T705A does not preferentially localize to the periphery (Fig 5B–D), in agreement with reduced surface expression shown in surface biotinylation experiments. We compared these distributions by evaluating their peak intensity near the cell periphery, a means of estimating the extent of membrane localization, as the peripheral peak corresponds to the surface localization for membrane proteins such as the Na+/K+-ATPase (Fig 5D). Inhibition using only GAK inhibitor 12i IVAP-1967 had a smaller effect on Atp1a3 distribution, although not statistically significant (Fig 5D), while dual inhibition caused reduction in Atp1a3 localization to the cell periphery. This set of experiments support the notion that phosphorylation of Atp1a3 by NAK family kinases is necessary for its surface expression.

Phosphomutant Atp1a3 accumulates in endosomal compartments in neurons

In order to examine localization of Atp1a3 in neurons, we co-transfected GFP-tagged Atp1a3 WT or TA with DsRed into rat hippocampal primary cultures. Atp1a3 WT showed typical surface distribution of Atp1a3 along the surface of dendrites and at dendritic spines, as previously reported (Blom et al, 2016) (Fig 6A). In contrast, the Atp1a3 TA phosphomutant formed clusters of large puncta in dendrites (Fig 6A). We repeated this experiment with HA-tagged Atp1a3 WT and TA co-transfected with GFP and found similar results (Fig S5A). Interestingly, HA-tagged TD showed diffuse distribution, but did not appear to localize to the surface of dendrites or to dendritic spines, consistent with ER retention (Fig S5A). To confirm that Atp1a3 TD is retained in the ER, we co-expressed HA-tagged Atp1a3 TD with either mCherry-tagged ER-3 (calreticulin) or mCherry-tagged Golgi-7 (β1,4-galactosyltransferase 1) in rat hippocampal neurons. We observed that while there was a high amount of co-localization of TD with mCh-ER3 in the ER, there was no apparent co-localization with the mCh-Golgi-7 marker (Fig S5B), supporting its retention in ER. We did not see co-localization with endogenous LAMP1, even when lysosomal degradation is inhibited by a lysosomal inhibitor cocktail (200 μM chloroquine, 200 μM leupeptin, and 10 mM NH₄Cl) for 1 h, indicating that lack of phosphorylation is not targeting NKA to lysosomes (Fig S5C).

To determine at which step of the endocytic trafficking pathway Atp1a3 TA-containing pumps were being retained, we used endogenous stainings or co-transfections with different intracellular membrane compartment markers in rat hippocampal neurons expressing Atp1a3 WT and TA. We found that a subset of puncta in WT and in TA mutants co-localize with endogenous stainings for a sorting endosome marker, early endosome antigen 1 (EEA1) (Fig 6B), lower magnifications of these neurons are shown in Fig S6A and B). We quantified the number of Atp1a3 puncta along the length of dendrites, imaging dendrites with comparable thickness. The density of intracellular Atp1a3 accumulations in dendrites was significantly higher in TA (0.45 ± 0.10 puncta/μm) when compared to WT (0.11 ± 0.04 puncta/μm) (P = 0.017, n = 7 cells each). We observed substantial co-localization with Atp1a3 WT and Atp1a3 TA; however,
the percentage of puncta that co-localize with EEA1 was not significantly different (25 ± 14% for WT and 39 ± 8% for TA). In addition, we found that co-transfections of Atp1a3 WT or Atp1a3 TA with markers for early endosomes (mCh-Rab5), recycling endosomes (mCh-Transferrin receptor; mCh-TRF), and late endosomes (mCh-Rab7a) all showed some degree of co-localization, indicating that Atp1a3 trafficking is not fully dependent upon T705 phosphorylation (Fig 7A). When we quantified the percentage of Atp1a3 WT or TA puncta co-localization with these endosomal markers, there were no differences observed in mCh-Rab5 (in %: WT 21.7 ± 4.2 (n = 3 cells); TA 24.3 ± 8.3 (n = 5 cells); P = 0.83), mCh-Rab7a (in %: WT 44.1 ± 6.4; TA 43.9 ± 10.5; P = 0.98, n = 5 cells, each), or mCh-TRF (in %: WT 50.8 ± 7.9; TA 31.3 ± 8.2; P = 0.13, n = 5 cells, each). Together with endogenous EEA1 stainings, these data indicate that similar percentage of Atp1a3 containing intracellular compartments co-localize with endocytic pathway markers; however, significantly more intracellular puncta are found in the TA mutant, specifically. Collectively, these data suggest that if Atp1a3 T705 cannot be phosphorylated, the NKA subsequently cannot be efficiently redirected from sorting endosomal compartments to the plasma membrane, supporting its functional importance.

Since our data indicate a reduced surface expression of phosphomutant Atp1a3, we tested whether phosphomutants are functional by making use of ouabain (Oua), a cardiac glycoside known to inhibit the Na’/K’-ATPase. We generated ouabain-resistant (OuaR) mutants of Atp1a3 by introducing Q108R and N119D mutations (Price & Lingrel, 1988) to all of the Atp1a3 phosphomutants. OuaR WT, TA, or TD mutants were co-transfected with either YFP-β1 or YFP-β2 into COS7 cells and treated daily with 5 μM ouabain to observe survival and recovery. Only cells that have integrated exogenous cDNA encoding NKA ouabain resistance into their genome are able to survive ouabain selection (Clapcote et al, 2009). Within 48 h of ouabain treatment, COS7 cells transfected with OuaR WT and YFP-β1 or YFP-β2 began to assume normal growth, enough to be passaged. However, none of the other OuaR phosphomutants were able to recover, and by the end of one week of ouabain treatment, 80–90% of all cells were dead and past recovery consistently, from three independent experiments. These observations suggest that the phosphorylation state of Atp1a3 T705 is critical for its function.

Na’/K’-ATPase function is affected in GAK NexCre conditional knockout mice

We next investigated the role of GAK in a knockout model, with a focus on putative substrate-linked phenotypes. We crossed Gak<sup>tm2Legr</sup> (Gak<sup>lox</sup>) mutant mice with mouse lines expressing Cre recombinase in different neuronal subsets. We observed that conditional knockout of GAK in Drd1a-expressing striatal neurons (Tg(Drd1a-Cre)<sup>yTos2Gsat</sup>) or in midbrain dopaminergic neurons (Dat<sup>hRES<sup>CRO</sup></sup>) resulted in embryonic lethality. Curiously, conditional knockout of GAK in Neurod6<sup>tm1Cre<sup>kan</sup></sup> (Ne<sup>Cro</sup>) mice, specifically in the excitatory pyramidal neurons of the neocortex and hippocampus, resulted in viable and healthy offspring with no discernible behavioural or physical phenotypes.

Expression of GAK protein is reduced in Western blot analysis of neocortical lysates from mutant Nex<sup>Cro</sup>/GAK<sup>lox/lox</sup> mice (F/F) compared to control Nex<sup>Cro</sup>/GAK<sup>lox/+</sup> mice (F/+). We generated ouabain-resistant (OuaR) mutants of Atp1a3 by introducing Q108R and N119D mutations (Price & Lingrel, 1988) to all of the Atp1a3 phosphomutants. OuaR WT, TA, or TD mutants were co-transfected with either YFP-β1 or YFP-β2 into COS7 cells and treated daily with 5 μM ouabain to observe survival and recovery. Only cells that have integrated exogenous cDNA encoding NKA ouabain resistance into their genome are able to survive ouabain selection (Clapcote et al, 2009). Within 48–72 h of ouabain treatment, COS7 cells transfected with OuaR WT and YFP-β1 or YFP-β2 began to assume normal growth, enough to be passaged. However, none of the other OuaR phosphomutants were able to recover, and by the end of one week of ouabain treatment, 80–90% of all cells were dead and past recovery consistently, from three independent experiments. These observations suggest that the phosphorylation state of Atp1a3 T705 is critical for its function.

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Discussion

In this study, we utilized a chemical genetics method with tandem mass spectrometry to identify a number of direct GAK substrates and their GAK phosphorylation sites. Among the substrates...
identified, we validated the sodium potassium pump α3-subunit Atp1a3 as a GAK target at the novel site T705. We further show that the Atp1a3 T705 site is important in NKA trafficking and function and that its function is impaired in a GAK conditional knockout model.

GAK, also called DNAJ26, is a member of the J-domain–containing protein family also called heat-shock cognate 40 (HSP40s). J-domain proteins bind heat-shock 70 kD proteins (HSP70s) and act as co-chaperones during ubiquitous processes including protein folding and degradation. It is important to note that GAK is the only protein in mammals that contains both a kinase and a J-domain (Kampinga & Craig, 2010). An intriguing speculation is that one function of the GAK domain is to complement its J-domain roles by phosphorylating proteins targeted by HSP70, such as the Na+/K+ -ATPase (Riordan et al, 2005) during trafficking of cargo. GAK’s role in phosphorylating the μ-subunits of the adaptor proteins AP-1 and AP-2 is well characterized (Umeda et al, 2000; Korolchuk & Banting, 2002; Kametaka et al, 2007), along with its role in clathrin uncoating (Zhang et al, 2005b; Lee et al., 2005, 2006).

NAK family kinases are conserved from yeast to mammals. In yeast, loss-of-function mutations of the NAK family kinases prk1p and ark1p cause accumulations of actin-associated endocytic vesicles that cannot traffic further to other membrane compartments (Sekiya-Kawasaki et al, 2003). In mammals, AAK1 regulates endocytic trafficking (Conner & Schmid, 2002) and trafficking of cell surface receptors. AAK1 regulates notch receptor recycling and signalling (Gupta-Rossi et al, 2011), and neuregulin-1/Erbb4 signalling by altering Erbb4 trafficking (Kuai et al, 2011). In our loss-of-function analysis enabled by specific GAK and AAK1/BIKE inhibitors, we find that there is a significant reduction in NKA’s peripheral distribution in COS7 cells. This finding is in agreement with reduced

Table 2. Intrinsic property values of neurons prior to ouabain application.

|                     | F/+         | F/F         | P-value |
|---------------------|-------------|-------------|---------|
| RMP (mV)            | −51.5 ± 1.3 | −54.4 ± 1.4 | 0.14    |
| Input resistance (MΩ) | 203.6 ± 49.5 | 220.4 ± 52.8 | 0.75    |
| Current injection (pA) | 35.8 ± 4.4  | 37.7 ± 6.0  | 0.81    |

GAK regulates Na⁺/K⁺-ATPase Lin et al. https://doi.org/10.26508/lsa.201800118 vol 1 | no 6 | e201800118 11 of 20
phosphomutant surface expression and increased presence of NKA in intracellular compartments. Therefore, it is possible that multiple members of the NAK family, including GAK and AAK1, could play complementary roles in receptor recycling via phosphorylating cargo proteins. Future experiments involving knockdown of GAK, AAK1, and possibly other NAK family members in neuronal cultures followed by evaluation of Atp1a3 trafficking would provide further support for their role in Atp1a3 trafficking.

In conjunction with known functions of NAK family kinases in membrane trafficking, we propose a simple model whereby GAK phosphorylates cargo proteins such as Atp1a3 and Sipa1L1 during or following endocytosis, with this phosphorylation being essential for efficient progression of Atp1a3 through subsequent trafficking steps, particularly for recycling back to the plasma membrane (Fig 8A). Previous work implicated PKA and PKC kinases in regulating NKA recycling in COS-1 cells (Kristensen et al, 2003). Our study demonstrates that without NAK family kinases or Atp1a3 T705 phosphorylation, NKA trafficking and function are impaired.

Importantly, due to the overlapping nature of substrate specificity among NAK family kinases, we cannot conclude that the identified putative substrates (Table 1) are phosphorylated by GAK and/or other NAK family kinases in organisms. In addition, all putative phosphorylation sites need to be validated by kinase assays.

**Regulation of sodium potassium pump trafficking by NAK family kinases**

For proper functioning of the sodium potassium pump, the pump needs to be correctly distributed to the plasma membrane, a function that is regulated by kinases. NKA phosphorylation by PKA has been shown to increase cell surface expression of NKA in mammalian kidney collecting duct cells (Vinciguerra et al, 2003), while in human skeletal muscle cells, insulin activity recruits NKA to the surface via phosphorylation by ERK1/2 kinases (Al-Khalili et al, 2004). Here, we show that a novel site at Atp1a3 T705 is phosphorylated by GAK, and other NAK family kinases, and is critical for Atp1a3 localization and pump function. A putative phosphomimetic mutant, Atp1a3 T705D, demonstrated ER retention, suggesting that successful NKA exit from the ER is reliant on this site not being phosphorylated. However, a non-phosphorylatable Atp1a3 T705A mutant demonstrates that once the pump has left the ER, phosphorylation at this site is necessary for the pump to be sorted to the plasma membrane, as phosphomutant Atp1a3 T705A has reduced surface expression. Increased total Atp1a3 levels in GAK knockout mice indicate alterations in NKA function, possibly reflecting NKA levels increased in response to reduced functionality.

The NKA can be regulated by either a change in intracellular Na+ concentration or a change in Na+ affinity (Toustrup-Jensen et al, 2014). We see an increase in pT705 levels during TTX treatment. TTX blockade of voltage-gated Na+ channels, while not changing RMP, would inhibit Na+ influx, resulting in a lower intracellular Na+ concentration. T705 phosphorylation may have a function in regulation of NKA in response to lower intracellular Na+ concentrations and reduced firing, perhaps facilitating its intracellular trafficking.

**GAK in mouse models**

In NexCre-mediated GAK conditional knockout mice, we observed no overall defects in either RMP or neuronal excitability. This contrasts with the embryonic or neonatal lethality observed when GAK is deleted in all neurons (Lee et al, 2008), dopaminergic neurons, or Drd1a-expressing medium spiny neurons in the striatum. This differential effect could be due to a compensatory mechanism present in pyramidal neurons, such as other NAK family kinases.

**GAK and ATP1A3 in neurodegenerative disease and aging**

GAK has been identified as a gene associated with Parkinson’s disease in several genome-wide association studies screens (Pankratz et al, 2014). Here, we show that a novel site at Atp1a3 T705 is phosphorylated by GAK, and other NAK family kinases, and is critical for Atp1a3 localization and pump function. A putative phosphomimetic mutant, Atp1a3 T705D, demonstrated ER retention, suggesting that successful NKA exit from the ER is reliant on this site not being phosphorylated. However, a non-phosphorylatable Atp1a3 T705A mutant demonstrates that once the pump has left the ER, phosphorylation at this site is necessary for the pump to be sorted to the plasma membrane, as phosphomutant Atp1a3 T705A has reduced surface expression. Increased total Atp1a3 levels in GAK knockout mice indicate alterations in NKA function, possibly reflecting NKA levels increased in response to reduced functionality.

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In addition, AAK1 is associated with Parkinson's disease in a genome-wide association study (Latourelle, 2009). GAK is shown to interact with α-synuclein in a pathway involved with PD pathogenesis (Dumitriu et al, 2011) as well as to form part of a binding complex with another known PD-risk gene leucine-rich repeat kinase 2 (LRRK2) (Beilina et al, 2014). In a Drosophila model of PD, the GAK ortholog auxilin has been shown to underlie locomotor defects along with dopaminergic neuron loss (Song et al, 2017). There is also evidence that a number of PD-associated genes, including LRRK2 and GAK, disrupt protein trafficking and degradation via the endosomal pathway as a consequence of age-related pathophysiology (Perrett et al, 2015).

Following neuronal activity, intracellular sodium clearance is mainly attributed to the α3-subunit (Azarias et al, 2013). Autosomal dominant mutations in ATP1A3 have been linked to rapid-onset dystonia parkinsonism (de Carvalho Aguiar et al, 2004; Bottger et al, 2011), alternating hemiplegia of childhood, and CAPOS syndrome, with more than 80 different disease-associated mutations reported, many of which target ion binding sites (Clausen et al, 2017). In addition, studies into neurodegenerative disease mechanisms have shown ATP1A3 deficits in a number of cases. In Alzheimer's disease (AD), there is reduced expression of Atp1a3 but not Atp1a1 in the frontal cortex of AD patients (Chauhan et al, 1997) as well as impaired NKA activity (Hattori et al, 1998). In AD, amyloid-β oligomers form unique assemblies called amylospheroids that target Atp1a3 and impair α3-containing NKA activity, leading to presynaptic calcium overload and neurodegeneration (Ohnishi et al, 2015). In PD, α-synuclein assemblies also target Atp1a3 by sequestering α3-containing NKA into clusters, ultimately affecting the sodium gradient (Shrivastava et al, 2015). These data support the possibility that deficiencies in GAK function in phosphorylating and regulating Atp1a3 may contribute to PD pathogenesis.

Our study uncovers novel roles for GAK in neurons, advancing our understanding of GAK function in cells. The conserved substrates and phosphorylation sites that we report will enable exploration of related functions in other tissues. In addition, the phosphorylation sites can be used to measure GAK activity in different tissues, during development and in PD disease models.

**Materials and Methods**

**Mouse maintenance**

Breeding and experiments were performed under the Animals (Scientific Procedures) Act 1986 of the United Kingdom and approved by institutional ethical review. Mice were group-housed and maintained on a 12-h light/dark cycle, with food and water provided ad libitum. Each mouse strain was backcrossed into C57 Bl/6 (Jackson) for at least three generations. None of the experimental mice were immunocompromised. Both male and female mice
were used and randomly allocated to experimental groups. Developmental ages between embryonic day (E)16 and postnatal day (P)30 were used as indicated in the figure legends. 

GAK (B6;129S6-Gak<sup>tm2Lee</sup> MMRRC stock JAX:36793) (Lee et al, 2008), Dat<sup>cre</sup> (B6.SJL-Slc6a3<sup>tm1.Cre</sup>8Mim/J) (IMSR Cat. No. JAX:006660, RRID: IMSR_JAX:006660) (Backman et al, 2006), and Thy1-YFP (Thy1-YFP) were obtained from Jackson Laboratories. Neurobasal Media (Invitrogen). Half of the media was replaced with fresh about every four days to maintain cultures until use.

For experiments, NexCre homzygous males (Nex<sup>Cre</sup>/Nex<sup>Cre</sup>) were bred with GAK homozygous (GAK<sup>F/F</sup>) females to generate experimental controls Nex<sup>cre</sup>/GAK<sup>F/+</sup> or mutants Nex<sup>cre</sup>/<sup>cre</sup> GAK<sup>F/+</sup> (F/F). All mice were bred and genotyped as recommended by Jackson Laboratories.

**Isolation of primary cells**

For rat primary cultures, E18 embryos of either sex from Sprague–Dawley rats (Charles River Labs) were used. After removal of the meninges, cortices and hippocampi were dissected, separately pooled, and mechanically triturated. Dissociated neurons were then plated in plating medium containing 10% foetal bovine serum (Hyclone), 0.45% dextrose, 0.11 mg/ml sodium pyruvate, 2 mM glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in modified Eagle’s medium. Hippocampal neurons were seeded onto 18-mm glass coverslips coated with 0.06 mg/ml poly-D-lysine (Sigma-Aldrich) and 0.0025 mg/ml laminin (Sigma-Aldrich) at a density of 0.3 × 10<sup>6</sup> cells per well. Neurons were seeded onto either 6-well or 12-well plates coated with poly-D-lysine/laminin at a density of 0.3 × 10<sup>6</sup> cells per well. After 4 hours, plating medium was replaced with maintenance medium containing 1× B27 (Invitrogen), 100 units/ml penicillin and 100 mg/ml streptomycin, 2 mM glutamine and 12.5 μM l-metatamente (K) cDNA (Source Bioscience) were cloned into HA-pRK5 by PCR cloning according to manufacturer’s instructions. Cells were collected for Western blot analysis 48 h post-transfection.

**Tissue culture cells**

HEK 293T cells and COS7 cells were maintained in DMEM (Gibco) supplemented with 10% FBS and 1% penicillin–streptomycin. Transfections were performed using Xtremegene 9 (Roche) according to manufacturer’s instructions. Cells were collected for Western blot analysis 48 h post-transfection.

**Plasmids and cloning**

GAK mouse (Open Biosystems) FL and kinase domain-only (K) cDNA were cloned into a pRK5 mammalian expression vector containing an N-terminal hemagglutinin (HA) tag (HA-pRK5) by PCR cloning using 5’ Sall and 3’ Noti restriction sites and the following oligos:

- GAK Sall Fwd: CATCGTGTCGACATGCGTCGAGCTG
- GAK Noti Rev: TCGAAGGGCCGGCTCAGAAAGGAGGGCTCGAGG

GAK<sup>1-400</sup> Noti Rev: ACAGTTCCGCGCGCTTACGATTGACAGAGACTGAT

All GAK mutants were generated by site-directed mutagenesis using QuickChange (Agilent Technologies) and the following oligos:

- GAK K69R (KD) Fwd: GCAGAGAGGATGATGATGCGAGCTACTATCC
- GAK K69R (KD) Rev: GGATGAACTGCTTACATCCTGAC

GAK inhibitors

The mCh-ER3 (plasmid #55041; Addgene) and mCh-Golgi7 (plasmid #55052; Addgene) plasmids were gifts from Michael Davidson. The mCh-Rab5 (plasmid #49201; Addgene) and mCh-Rab7A (plasmid #55052; Addgene) plasmids were gifts from Michael Davidson. The mCh-ER3 (plasmid #55041; Addgene) and mCh-Golgi7 (plasmid #55052; Addgene) plasmids were gifts from Michael Davidson.
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Immunostaining

Cultured hippocampal neurons on glass coverslips were fixed for 10 min at RT with 4% paraformaldehyde/sucrose in PBS, permeabilized for 10 min RT with 0.1% Triton-X100 in PBS, and then blocked for 1 h RT with 10% normal goat serum in PBS. Cultures were incubated with primary antibodies in blocking buffer at 4°C overnight. After three washes in PBS, cultures were labelled with fluororecytometry are rat anti-HA (1:500; Roche), mouse anti-EEA1 (clone 14: 1:250; BD Transduction Laboratories), rabbit anti-LAMP1 (ab24170, 1:500; Abcam), and mouse anti-c-Myc (1:500; Thermo Fisher Scientific). Fluorescent secondary antibodies with minimal cross-reactivity to other species were obtained from Invitrogen or Jackson ImmunoResearch.

Western blotting

For drug treatment experiments, DIV14–15 cultured cortical neurons in a 12-well plate were used. Each well was treated for 1 h in culture medium. Neurons were directly lysed with 1× sample buffer (4× stock; Invitrogen) containing 400 mM DTT. Lysates were denatured for 10 min at 95°C.

For CIP dephosphorylation experiments, DIV15 rat cortical cultures in 60-mm dishes were treated for 1 h with either DMSO, 0.5 μM OA, or 10 μM cyclosporin A and lysed in lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40 [Igepal], 10% glycerol, 1× protease inhibitor cocktail [Roche]). Lysates were left to solubilize on ice for 10 min and then clarified by centrifugation for 10 min at 20,000 g. An aliquot of input was reserved as needed, and the remaining supernatant was incubated with 30–40 μl of HA beads (clone 3F10; Roche) for 2 h rotating at 4°C. The beads were washed three times in IP buffer before proteins were eluted in 2× sample buffer and denatured for 10 min at 95°C.

Protein samples were subjected to SDS–PAGE separation on 4–12% gradient gels according to manufacturer’s protocol (Invitrogen). Resolved proteins were transferred to polyvinylidene fluoride membrane, blocked in either 5% non-fat dry milk (Sigma-Aldrich) or 5% BSA (Sigma-Aldrich) for 1 h RT, and incubated overnight at 4°C with primary antibodies. Signal was detected using horseradish peroxidase (HRP)–conjugated secondary antibodies (Jackson) followed by a chemiluminescence reaction using Amersham ECL substrate (GE Healthcare). Chemiluminescent signal was detected using X-ray film (GE Healthcare). Densitometry was done using ImageJ, and values were analysed using t test.

Antibodies used in immunoblots include rat anti-HA (3F10 1: 5,000; Roche), mouse anti-α-tubulin (120,000; Molecular Probes), and mouse anti-Atplα3 (130,000; Thermo Fisher Scientific). GAK rabbit antibody was a gift from Lois Greene used at 1:10,000. Phosphoantibodies were generated by Covab. Atplα3 phospho-T705 antibody was generated against the mouse Atplα3 epitope TQRAGHRRL-phospho-S-DV (1:500 for Western blot). Horseradish peroxidase–conjugated secondary antibodies against mice, rats, and rabbits were used between 1:10,000 and 1:25,000 (Jackson ImmunoResearch).

Surface biotinylation

Cells were washed 2× with cold PBS and then incubated in 1× PBS containing 1 mg/ml sulpho-NHS biotin conjugate (Pierce) for 30 min rocking at 4°C. Cells were washed 2× to remove unbound biotin, then lysed in NP40 lysis buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 1% NP40 [Igepal], 10% glycerol, 1× protease inhibitor cocktail [Roche]), and 1× phosphatase inhibitor cocktail 3 (Sigma-Aldrich)), and centrifuged at 20,000 g for 10 min. After reserving an aliquot for input, the remaining supernatant was incubated with 30 μl of streptavidin beads (Pierce) for 1 h rotating at 4°C. The beads were washed 3× in lysis buffer, and surface proteins were eluted in 2× sample buffer for 10 min at 95°C.

Protein purification

GAK K constructs for protein purification, analog-specific (AS) and KD, were cloned into the pFastBac HT B vector (Invitrogen) by PCR cloning to contain N-terminal 6× His tag. His-tagged GAK-K-AS and GAK-K-KD baculovirus expression (Bac-to-Bac Baculovirus expression system; Invitrogen) was performed according to manufacturer’s protocol in insect cells.

Kinase assays

GAK assays: GAK was expressed in HEK 293T cells for 48 h maintained in 10% FBS, 5% pen/strep in Dulbecco’s modified Eagle medium. Cells were lysed with lysis buffer containing 1% Nonidet
P-40 (Igepal), 10% glycerol, 1 mM Na3VO4, 20 mM β-glycerol phosphate, 50 mM NaF, 1× complete protease inhibitor cocktail (Roche), 1× phosphatase inhibitor cocktail 3 (Sigma-Aldrich) in 20 mM Tris–HCl pH 8.0 and 150 mM NaCl. Lysates were incubated on ice for 30 min and centrifuged at 20,000 g for 15 min. Supernatant was precleared with IgG-Sepharose (GE Healthcare) for 30 min and incubated with Anti-HA Affinity matrix (clone 3F10; Roche) for 2 h at 4°C to immunoprecipitate HA-tagged GAK. Beads were washed twice with lysis buffer, once with lysis buffer containing 1 M NaCl for 10 min and once with lysis buffer for 10 min. After three additional washes with kinase reaction buffer (20 mM Tris–HCl pH 7.5, 10 mM MgCl2, 1 mM DTT, 1 μM cyclic AMP–dependent protein kinase inhibitor peptide and 1 μM OA), beads were incubated in a kinase reaction mixture for 30 min at 30°C. Reaction volume was 30 μl in addition to the bead volume. Either 0.5 mM ATP-γ-S (Sigma-Aldrich) or 0.5 mM of the analog ATP-γ-S (6-Bn-ATP-γ-S, 6-PhET-ATP-γ-S or 6-Furfuryl-ATP-γ-S from BioLog Life Science Institute) was included in the reaction. The reaction was immediately followed by a 1 h alkyllyation reaction at RT by adding 1.5 μl of 100 mM p-nitro mesylate (PNBM) per 30 μl of kinase reaction. The reaction was stopped by the addition of 4× sample buffer containing 400 mM DTT to a final concentration of 1×, and proteins on the beads were denatured at 95°C for 10 min. Supernatants were run on a Western blot. Thiophosphorylation was detected by anti-thiophosphate ester antibody 1:30,000 (Abcam).

Measurement of Atp1a3 distribution in COS7 cells using ADAPT Image software plugin

COS7 cells were split at confluency and GFP-tagged β1 was co-transfected with either HA-tagged Atp1a3 WT or T705A. After 48 h, cells were treated with either DMSO or 2 μM LP for 1.5 h. Cell media was then replaced for 30 mins with a serum-free media including either DMSO or LP. This was followed by the addition of 5 μM D2 or DMSO into wells for another 2 h. DMSO control, D2, LP or D2+LP conditions all received the same total volumes of DMSO solvent. LP treatment was done for 4 h total and 12i treatment for 2 h total (in serum-free conditions). Cells were fixed with 4% PFA/sucrose for 10 min at RT and stained using anti-HA antibody. β1 expression was visually confirmed in all imaged cells.

In vivo tissue processing

NexCre+/−;Gaklox/lox,Thy1-YFP and NexCre+/−;Gaklox/−,Thy1-YFP mice (P18–P20) were anaesthetized by intraperitoneal injection of 70–100 mg/kg ketamine + 10–20 mg/kg xylazine and perfused with ice cold PBS followed by 4% PFA. Brains were post-fixed in 4% PFA overnight at 4°C. Using vibratome sectioning, 50–100-μm-thick coronal sections were collected. Sections were mounted to glass slides using Fluoromount-G and imaged.

Electrophysiology

NexCre+/−;Gaklox/lox and NexCre+/−;Gaklox/− mice (P18–P22) were anaesthetized by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). Mice were killed by cervical dislocation. Brains were removed, the midline was cut down, and the cut surface was glued to the stage of the slicing chamber containing ice cold (~1°C) sucrose artificial cerebral spinal fluid (aCSF; composition in mM: 189 sucrose, 26 NaHCO3, 2.5 KCl, 5 MgCl2, 0.1 CaCl2, 1.2 Na2HPO4, 10 glucose), gassed with 95% O2–5% CO2. Sagittal slices (300 μm thick)
were prepared and transferred into an interface storage chamber containing aCSF (composition in mM: 125 NaCl, 3 KCl, 1 MgCl₂, 2 CaCl₂, 1.2 NaH₂PO₄, 26 NaHCO₃, 10 glucose) bubbled with 95% O₂–5% CO₂ at RT. Slices were left in the interface storage chamber for at least 1 h before use and were used up to 8 h after slicing.

Whole-cell patch clamp recordings were made from the somata of hippocampal CA1 pyramidal neurons using infrared differential interference contrast optics. Slices were constantly perfused with 95% O₂–5% CO₂ oxygenated aCSF at a flow rate of ~3 ml min⁻¹ at 31–32°C. Patch electrodes (3–7 MΩ) were pulled from borosilicate glass. Membrane currents were recorded using a Multiclamp 700B amplifier (Molecular Devices), sampled at 5 kHz, and low pass Bessel–filtered at 1 kHz. Compensations for slow and fast capacitive currents were performed. Data acquisition was controlled using Clampex 10.3.

Miniature excitatory postsynaptic currents were recorded at ~75 mV using an intracellular solution comprising the following (mM): 140 CsCH₃SO₄, 8 NaCl, 10 Hepes, 0.5 EGTA, 2 Mg-ATP, 0.3 Na-GTP, 5 QX-314, and 0.2% biocytin; osmolarity ~285 mosmol L⁻¹; pH 7.3 with CsOH. Miniature EPSCs were isolated with 10 μM gabazine and 1 μM tetrodotoxin (Tocris Chemicals). For firing studies, the intracellular solution comprised (mM) 120 K+-gluconate, 24 KCl, 4 NaCl, 4 MgCl₂, 0.16 EGTA, 10 Hepes, 4 K₂-ATP, and 0.2% biocytin, pH 7.2 adjusted with KOH.

Electrophysiological data analysis

Cells were rejected if input/access resistance or holding current changed by more than 20% of the initial value after establishing the whole-cell configuration. For miniature excitatory postsynaptic currents, 5 min of data was analysed from each viable cell, and post-synaptic currents were manually detected using MiniAnalysis software (v6.0.3, Synaptosoft). The amplitude and area detection threshold was set to five times greater than the root mean square of the baseline noise. For firing studies, data were analysed with pClamp10 software (Molecular Devices). Statistical analysis was performed using Origin 2017 software (OriginLab). Data were tested for normality using a Shapiro–Wilk test; significant criterion was α = 0.05. Mann–Whitney U test was used on non-normally distributed data.

Supplementary Information

Supplementary Information is available at https://doi.org/10.26508/is.201800118

Acknowledgments

We thank members of the Ullanir Lab for their feedback and thoughtful suggestions, Richard Li for his expertise in protein alignment and PYMOL modelling, Ian Rosewell and Sunita Varsani-Brown for their generation of the clustered regularity interspersed short palindromic repeats Atp1a3TT705A mouse, David Barry for help with ADAPT ImageJ plugin and light microscopy analysis, and Lucas Baltussen for manuscript preparation. We thank Dr Hjalmar Brismar for GFP-tagged Atp1a3 plasmid. This work was supported by the Francis Crick Institute which receives its core funding from Cancer Research UK (FC001201), the UK Medical Research Council (FC001201), and the Wellcome Trust (FC001201).

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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