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In vivo Brain GPCR Signaling Elucidated by Phosphoproteomics

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Summary:

Obtaining a systems view of G protein-coupled receptor (GPCR) signaling in its native environment is the key in development of GPCR therapeutics with fewer side effects. Using the kappa opioid receptor (KOR), a model GPCR, we employed high-throughput phosphoproteomics to investigate downstream signaling induced by structurally diverse agonists in five mouse brain-regions. Through quantification of 50,000 different phosphosites, this approach yielded a systems view of KOR in vivo signaling, revealing novel mechanisms of drug action. Pathway-selective agonists elicited differential dynamic phosphorylation of synaptic proteins, linking GPCR signaling to modulation of brain functions. We also discovered enrichment of mTOR pathway in agonists associated with aversion, a side effect. Consequently, mTOR inhibition during KOR activation abolished aversion, while preserving therapeutic analgesic and anticonvulsant effects. Our results establish high-throughput phosphoproteomics as a general strategy to investigate GPCR in vivo signaling, enabling prediction and modulation of behavioral outcomes.

The G protein-coupled receptors (GPCRs) superfamily contains some most prolific drug targets in numerous therapeutic areas, including cancer¹ and cardiac² and neurological³ diseases. Stimulation of a single GPCR, such as the kappa opioid receptor (KOR), often activates parallel pathways, each leading to distinctive physiological outcomes. For example, KOR triggers beneficial analgesic, anti-pruritic⁴ and anticonvulsive/antiepileptic⁵ signaling⁶ on one hand and undesirable dysphoria or aversion and psychotomimetic effects on the other^{6,7}. (Fig. 1a). Recently, some KOR agonists were found to be devoid of aversion in mice (non-aversive agonists) in contrast to the traditional agonists (aversive agonists). Functional selectivity, a GPCR signaling paradigm derived from *in vitro*^{8,9} and structural¹⁰ experiments, was used to explain this phenomenon^{11,12}. However, KOR functional selectivity in cells transfected with KOR is not directly translatable to cells naturally

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expressing KOR¹³ or into behavioral effects *in vivo*¹⁴. Especially the complexity of brain circuitry hinders the *in vitro-in vivo* correlation through heterogeneity at cellular, pathways, and protein levels¹⁵. Thus, it is imperative to develop an unbiased approaches that directly investigate GPCR and specifically KOR signaling *in vivo*, bridging the gap between biochemical and structural approaches, and the largely elusive downstream signaling consequences of GPCR activation in the brain.

Developments in mass spectrometry (MS)-based proteomics in general, and phosphoproteomics in particular, now enable the near comprehensive characterization of proteomes and tens of thousands of phosphorylation events^{16–19}. Application of MS to study GPCR signaling has been limited to assaying the phosphorylation state of a single receptor²⁰ or small-scale phosphoproteomics on *in vitro* cell lines^{21–23} or *ex vivo* cells²⁴. Very recently, we described the EasyPhos technology, which streamlines phosphoproteomics to such an extent that hundreds of phosphoproteomes can be measured in a short time and at uncompromised depth of coverage, enabling time course studies of dynamic and signaling events *in vivo*^{25,26}. Here we apply this technology along with behavioral and pharmacological investigations to attain a systems view of KOR signaling induced by pathway-selective agonists in temporally and spatially resolved areas of the mouse brain.

Brain phosphoproteomic architecture

We intracisternally (i.c.) administered three aversive and two non-aversive ligands according to previously established dosages⁵ (Fig. 1b, **method**). After short (5 min) and long (30 min) intervals post-injection, we dissected four anatomical brain regions that express KOR at different levels (striatum, hippocampus, cortex and medulla oblongata) and one without observable KOR expression (cerebellum) (Fig. 1c). In total, including biological replicates and follow up experiments we measured over 300 single-shot label-free phosphoproteomic experiments using the high-throughput EasyPhos platform²⁵ (Fig. 1d). Together, this yielded more than 60,000 different identified phosphosites, mapping to about 6,700 brain proteins. This is the most comprehensive coverage of any organ phosphoproteome reported to date, underscoring the diversity and importance of phosphorylation-based regulation in the brain (Fig. 1e). To obtain a general overview of the architecture of the brain phosphoproteome, we performed principal component analysis (PCA), revealing that the phosphoproteomes of each brain region clustered tightly with others of the same region. This indicates that each region possesses a unique phosphoproteome signature. Furthermore, brain regions that shared a similar developmental origin exhibit similarities in their phosphoproteome (Component 2 of the PCA in Extended Data Fig. 1a). The region-specific nature of the phosphoproteome is partly driven by the underlying differences in the proteomes, as proteins highly expressed in one brain region (e.g. striatum), often also yielded prominent phosphopeptides in the same region (Extended Data Fig. 1b, c). In particular, expression levels of kinases²⁷ (the ‘kinome’) correlated significantly with the abundance of phosphopeptides containing the corresponding linear motifs (Extended Data Fig. 1d). Thus, proteome and kinase expression in different regions partially shape the brain phosphoproteome. We observed that 85% of synaptic proteins were phosphorylated, which is of particular interest given previous reports of the importance of phosphorylation in the regulation of synaptic plasticity²⁸.

Approximately 50,000 of the identified phosphorylation sites were quantifiable (Fig. 1f). As established in our recent studies using EasyPhos, reproducibility between biological replicates was robust with an average Pearson Correlation of around 0.85 (Extended Data Fig. 2). Injection of the reference KOR aversive agonist, U-50,488H (“U-50”) significantly perturbed about 5% of these sites, with differing ligand perturbation patterns in distinct brain regions (Fig. 1g). After 5 min U-50 stimulation we observed maximum perturbation in striatum (1,000 sites), and progressively less in the order of hippocampus > cortex > medulla oblongata, in line with the expression level of KOR in the respective brain regions²⁹. In the cerebellum, where KOR is not detectable, there were hardly any regulated sites, providing a strong validation of our technology to the brain (Fig. 1g). Overall, these results demonstrate that phosphoproteomics enables observation of KOR agonists activation in a brain region-specific fashion.

KOR signaling is brain region-specific

Next, we analyzed ANOVA significant, spatially and temporally U-50-regulated sites. U-50 activates inhibitory G proteins (Gi/o) and induces internalization of KOR through β -arrestin 2 recruitment³⁰. Inhibition of Ca^{2+} channels modulates release of neurotransmitters such as GABA, dopamine and glutamate, and negatively regulates all three neurotransmitter mediated signaling. Accordingly, our bioinformatics analysis of regulated phosphosites³¹ revealed significant enrichment of Gene Ontology (GO) terms such as “inhibition of adenylate cyclase activity by G-protein signaling pathway”, “positive regulation of receptor internalization” and “regulation of neurotransmitter secretion”. This unbiased approach also highlighted mechanisms not yet linked to KOR activation, such as “RNA splicing” (Extended Data Fig. 3a). Interestingly, the phosphorylation states of numerous membrane proteins, for instance, voltage-gated sodium channels, neurotransmitter transporters, are altered by KOR activation (Extended Data Fig. 3b)

Like the basal brain phosphoproteome, phosphosites clustered tightly by replicates. Interestingly, the PCA map was dynamic in time, with the relative positions of cortex, medulla, hippocampus and striatum changing drastically between the time points (Fig. 2a, b). In the first component, striatum deviated most from the other regions at the 5-min interval, whereas cortex diverged mostly at 30 min, suggesting that U-50 perturbation is most profound in the striatum at early time points and in the cortex at later time points. Striatum is a part of the mesolimbic pathway and the cortex is a part of the mesocortical pathway, both representing the two most prominent pathways in KOR signaling (also reflected in the relatively high KOR expression in both tissues). Unlike *in vitro* cell model systems, each brain regions comprise of elaborated connections of heterogeneous neurons. In this complex background, we also discovered U-50 mediated KOR signaling to be highly region-specific. For example, we found that U-50 regulated sites in striatum were specific to this region, a result that also held for the other brain regions (Fig. 2c, Extended Data Fig. 4).

At the level of known, functional sites, we observed de-phosphorylation of dopamine- and cAMP-regulated neuronal phosphoprotein (DARPP-32) on pS97 and of tyrosine kinase Src on pS17 exclusively in striatum. Raf1 was dephosphorylated at pS259 only in cortex and ERK1 was phosphorylated at pT203/pY205 only in hippocampus (Fig. 2d). We further

investigated the regional specificity of ERK1 phosphorylation using immunohistochemistry. This observation validates the phosphoproteomics findings and reveals that elevated ERK1 phosphorylation is specifically located on hippocampal mossy fibers (Fig. 2e).

In contrast to the basal phosphoproteome, which was partly shaped by the proteome, and particularly the kinome (Extended Data Fig. 1), no correlation between U-50 regulated phosphorylation events and the region-specific proteome or basal phosphoproteome was observed (Extended Data Fig. 5). This implies that region-specific regulation is not just a function of expression levels of kinases and substrates, but that it results from more complex factors such as differing protein-protein interaction networks, neuronal contacts, or the position of the tissue in neuronal circuitries. Indeed, mapping the U-50 regulated phosphorylation events onto known interaction networks (**Methods, Interaction network analysis**), highlighted the signaling pathways relevant to the specific physiological functions of each brain region, including “neurotransmitter secretion”, “dopaminergic synapses” enriched in striatum, “axon guidance”, “long-term potentiation” and “calcium signaling” enriched in hippocampus, which is not the case for the basal phosphoproteome (Extended Data Fig. 6).

Next, we contrasted signaling of the initially investigated aversive agonist U-50 with that of 6’GNTI³², as an example of a KOR non-aversive agonist. Depending on the brain region and time point, U-50 and 6’GNTI shared between 30 to 50% of regulated sites. Substantial differences were apparent in the first component of a PCA between U-50 and 6’GNTI after 5 min stimulation in striatum and hippocampus, but not in medulla oblongata and cortex. After 30 minutes of stimulation the ligand differences were greatly reduced in striatum, but were more pronounced in other regions, especially the cortex (Extended Data Fig. 7). Since the i.c. injected doses of each ligand are the same across brain regions and time points, these region- and time-dependent changes between functionally biased ligands may reflect the position of the regions in distinct brain circuitry.

KOR signaling in the striatal synaptic phosphoproteome

Some KOR agonists induce aversive states, primarily through activation of medium spiny neurons in the nucleus accumbens (NAc) of ventral striatum^{12,33}. To elucidate the molecular basis for this profile, we employed three aversive agonists (U-50, HS665 and RB64) and two non-aversive ligands (6’GNTI, HS666). Among these agonists, HS665 and HS666 are characterized recently³⁴, providing a framework for phosphoproteomic-based ligand characterization in the future. Bioinformatic analysis results showed that the regulated phosphoproteins of the aversive agonists, but not the non-aversive agonists were significantly enriched for “potassium channel complex”, “cell junction” and “excitatory synapse” (**Methods Annotation Enrichment Analysis**, Extended Data Fig. 8a). This prompted us to focus on differential phosphorylation of synaptic proteins, particularly because dynamic phosphorylation of synaptic proteins in striatum affects synaptic plasticity²⁸ and membrane trafficking³⁵.

Overall, large-scale dynamic phosphorylation changes on synaptic proteins were present at the 5 min interval and ebbed away at the 30 min interval (Extended Data Fig. 8b). The most

prominent dynamic phosphorylation changes mediated by aversive agonists, but not by non-aversive agonists involved synaptic proteins associated with dopaminergic, glutamatergic, and GABAergic signaling and synaptic vesicle release. This included a downregulation of the phosphorylation state of the NMDA receptor and many of its scaffolding proteins, dynamin (Ser851 on Dnm1), members of the SNARE complex, along with a multitude of ion channels (Fig. 3a).

We were especially intrigued by DARPP-32, a downstream effector of dopaminergic signaling that is also regulated by glutamatergic signaling. DARPP-32 is a potent inhibitor of protein phosphatase 1 (PP1) and plays an important role in synaptic plasticity³⁶. Dynamic phosphorylation of DARPP-32 in general and specifically Ser97 is regulated by various drugs of abuse^{37,38}, and is correlated with nuclear accumulation of DARPP-32 and the control of gene expression related to long-term synaptic plasticity³⁷. From the MS measurements, we found Ser97 to be specifically dephosphorylated two-fold at 5 min after application of the aversive KOR agonist U-50 in striatum. Immunohistochemistry clearly showed this de-phosphorylation in both caudate putamen (CPu) and NAc, the two main loci of striatum. This effect was specific to the aversive KOR agonists and to Ser97, whereas Ser192 was not regulated (Fig. 3c).

Other interesting ligand-directed dynamic phosphorylation events include cannabinoid receptor 1 (CB1). Phosphorylation level of CB1 at Ser317 was shown to correlate with CB1 activity, while mounting evidence links CB1 activation with KOR activation^{39–41}. Our current data suggests that CB1 and KOR cross-talk may at least be partially regulated through dynamic phosphorylation of CB1 at Ser317 (Fig. 3a, c).

Among the kinases, downregulation of Src kinase at Ser74 by aversive, but not non-aversive agonists is noteworthy because of Src's known role in phosphorylating many of the above-mentioned substrates and regulating synaptic plasticity⁴². Other sites of interest include Ser7 of G protein gamma subunit 12, which is an immediate downstream effector of KOR and Ser1459 of the aforementioned NMDA receptor 2a subunit.

These examples illustrate the complexity and subtlety of the differential regulation of various signaling pathways in the nervous systems by these two ligand classes in the important context of the synapse.

Phosphatases participate in KOR striatal aversive signaling

The large-scale de-phosphorylation of synaptic proteins by aversive agonists raised the question if they specifically activated serine/threonine phosphatases. To test this hypothesis, mice were administered i.c. three functionally distinct phosphatase inhibitors: Fostriecin, a protein phosphatase 2A (PP2A) and 4 (PP4) inhibitor; Calyculin A, a PP1 and PP2A inhibitor; and Tautomycin, a selective PP1 inhibitor (Fig. 4). One hour post-treatment, mice received U-50, brains were dissected 5 min later, and with striatum processed for phosphoproteomic measurements as previously described.

Consistent with our hypothesis, we found 300 phosphorylation sites in the striatum for which de-phosphorylation was abolished in the presence of one or more phosphatase

inhibitors. This establishes that phosphatases play an important role in aversive agonist signaling. About half of these phosphatase-sensitive sites belong to synaptic proteins, in agreement with the importance of phosphatases in synaptic functions⁴³. In addition, Gene Ontology (GO) enrichment analysis of the proteins regulated by phosphatase revealed enrichment of “clathrin-dependent endocytosis”, “synaptic vesicle priming” and “small GTPase regulator activity”, suggesting that an aversive agonist pathway but not non-aversive agonist pathway may be involved in neurotransmitter release and membrane receptor trafficking (Extended Data Fig. 9).

Among the 300 phosphatase-sensitive sites, a minor cluster was insensitive to Tautomycin pre-treatment (Fig. 4a). Unlike the other two inhibitors, Tautomycin selectively inhibits PP1 but not PP2A, therefore these sites are most likely mediated by PP2A. In this group of sites, we were especially intrigued by the aforementioned phosphorylation of Ser317 of the CB1 receptor and Ser762 of the GABA_B receptor (Fig. 4b), both well-known GPCRs. Thus, phosphoproteomics can reveal additional mechanistic details in the complexity of *in vivo* GPCR signaling through the phosphorylation of co-expressed GPCRs. Together these results highlight the importance of phosphatases in GPCR signaling at synapses.

Aversion, not Analgesia or Anti-convulsion, is ablated by mTOR pathway blockade

The bioinformatic analysis of the sites differentially regulated by aversive and non-aversive agonists also revealed that the mammalian Target of Rapamycin (mTOR) signaling pathway was enriched in striatum at the 5 min interval and in cortex at 30 min (Fig. 5a, b). In striatum, mTOR was the most significantly regulated pathway, whereas in cortex it was among the top five. This interesting finding from our unbiased phosphoproteomics approach links to previous reports involving mTOR in ketamine-induced and fluoxetine (ProzacTM)-induced anti-depressant effects in a region-dependent manner and dysfunction of the mTOR pathway in pre-frontal cortex disorders of patients with major depressive disorders^{44–46}. Combined with our present observation that only aversive agonists induced mTOR signaling, this led us to hypothesize that pre-treating mice with an mTOR inhibitor could abolish the aversive behavior commonly induced by aversive KOR agonists.

We investigated the effects of pretreatment with Temsirolimus, an mTOR inhibitor at 8 mg/kg, on ‘conditioned place avoidance (CPA)’, a well-established behavioral model for assessing aversion in animals. Mice were pretreated for one hour either with Temsirolimus, an mTOR inhibitor, saline or DMSO controls before receiving U-50 and saline injection for each conditioning session (Fig. 5c). As expected, treatment with U-50 induced a highly significant response in the CPA assay, indicating that this aversive agonist induced aversive effects in mice. Pretreatment with the mTOR inhibitor, abolished the CPA induced by U-50 alone (Fig. 5c). This shows that mTOR blockade indeed suppressed the aversive effects of U-50 linked to KOR activation, which is in agreement with our hypothesis. Importantly, the anticonvulsive effects of U-50 as assessed by pentylenetetrazole tail-vein infusion (Fig. 5d) and the analgesic effects assessed in the acetic acid-induced writhing test (Fig. 5e) were unaffected by mTOR inhibition.

To probe the downstream molecular mechanism of mTOR activation, we found that the two KOR ligand classes differentially regulated phosphorylation sites of proteins involved in translation including Eif4b (Ser 504) and Rps6 (Ser240, Ser244) (Extended Data Fig. 10). A body of evidence already links mTOR-mediated alteration of protein synthesis with synaptic plasticity, learning and memory, specifically through long-term potentiation and depression.⁴⁷ Our present data specifically connects KOR activation to depressive/aversive behavior via the activation of mTOR, possibly through its effect on protein translation, and shows that this connection can be pharmacologically separated.

To investigate the upstream mechanism that activates mTOR, we used the neuroblastoma cell line (Neuro 2A) with stably expression of the mouse KOR. Cells were pretreated with vehicle or pertussis toxin (PTX) (200ng/ml) a specific inhibitor of the Gi/o signaling pathway, for 2 hours, followed by vehicle or U50 (10uM) for 30 min. Phosphoproteomic analysis of these cells revealed that mTOR signaling was the most enriched pathway in U-50, and U-50 + PTX ($p < 10^{-8}$), but not in PTX-treated cells alone, indicating that mTOR signaling is activated in a G protein-independent manner (Extended Data Fig. 11). Specifically, we discovered that Ser939 of Tsc2 was phosphorylated by U-50 treatment in a PTX insensitive manner (Extended Data Fig. 12). Phosphorylation of Tsc2 by Akt modulates mTOR signaling⁴⁸ and we found an additional group of Akt substrates to be influenced by U-50 that were also PTX-insensitive (Extended Data Fig. 13), among molecules which likely mediate G protein-independent signaling.

Along with the mTOR pathway, we also found the MAPK pathway to be enriched in U-50 and U-50 + PTX stimulated Neuron 2A cells. Early activators such as Sos1 and Raf1 were de-phosphorylated in a PTX-insensitive manner (Extended Data Fig. 12). Activation of the MAPK pathway has already been linked to KOR G protein-independent signaling pathways and KOR-mediated aversion^{11,12}. Thus, our current data establishes that both mTOR and the MAPK pathways are activated in a G protein-independent manner. This leads us to speculate that these two G protein-independent signaling pathways are activated in a similar fashion to activation downstream of the insulin receptor: MAPK through the Grb2-Sos1-Raf branch, and mTOR activation through the Akt-Tsc2 branch (Extended Data Fig. 12).

Discussion

This study offers a first systems view of *in vivo* brain GPCR signaling. Even though KOR-mediated signals derive from interaction of ligands with the same GPCR, in each brain region and time point, signaling pathways are channeled towards distinct physiological effectors. The basal phosphoproteome in different regions correlated with the abundance of the proteome and the kinome. This was not the case for the regulated phosphoproteome, reflecting the importance of additional factors, such as protein interaction networks, neuron-neuron contacts and brain circuitry in the makeup of the regulated phosphoproteome. This was apparent from signaling propagation to brain areas with minimal KOR expression at later time points. Such results emphasize that a more complex concept of GPCR signaling, which accounts for diversity of cell types and cell-cell communication in addition to *in vitro* concepts such as functional selectivity is necessary for a better understanding of the subtleties of *in vivo* GPCR signaling.

Our study has also established that the phosphoproteomic approach could disentangle beneficial analgesic, anticonvulsive effects from side effects such as aversion at the pathway level, and possibly use this knowledge to separate one from the other. This *in vivo* approach bypasses the need of *in vitro* characterization of ligands and minimizes the risk of *in vitro* to *in vivo* translation. Although Temsirolimus, the mTOR inhibitor used here is a FDA-approved chemotherapy agent, clinical application of these results would likely require a specific modulation of the mTOR network that results in the desired behavioral outcomes.

In conclusion, we propose phosphoproteomics combined with pharmacological tools and behavioral assessments as a general approach for studying GPCR signaling *in vivo*, combined with appropriate or cellular systems for detailed molecular mechanistic characterization of individual signaling pathways. Our approach bridges *in vitro*-based and purely behavioral studies, offering the opportunity to discover, without *a priori* assumptions, signaling pathways that can be pharmacologically manipulated to achieve specific therapeutic benefits.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. High-throughput phosphoproteomic of in vivo KOR-mediated GPCR signaling.

(a) KOR signaling activates analgesia while inducing side effects. (b) Five KOR agonists, including two non-aversive agonists and three aversive agonists were administered intracisternally to mice. U-50, a aversive agonist, served as the reference KOR agonist. (c) Drug-treated groups of mice were sacrificed after 5 (short) or 30 (long) minutes post-injection in each experimental group. Five brain regions were. (d) The single run EasyPhos workflow. (e) Out of ca. 50,000 quantifiable phosphosites, less than 5% of those sites were perturbed by the U-50 treatment. (f) Overall statistics of the brain phosphoproteome. (g) The magnitude of U-50 induced perturbation differs between brain regions: striatum has the most detectable perturbation with over 1,000 sites, whereas cerebellum has minor observable U-50 induced perturbation.

Fig. 2. Region-specific outcomes of U-50 induced activation of KOR.

(a) Principle Component Analysis (PCA) of normalized spatial and temporal U-50 ANOVA significantly regulated sites against their respective saline controls at the early 5 min time point. (b) Same as (a) at the late 30min time point. (c) Scatter plot showing striatum specific U-50 signaling: the x-axis denotes the log₂ intensity difference of striatum samples between U-50 and saline control; the y-axis denotes the log₂ intensity difference between all the striatum samples and that from the other brain regions. The U-50 regulated sites that are striatum specific fall on the diagonal as indicated by the blue shade, whereas U-50 regulated sites that are in common between all brain regions fall on the x-axis, as indicated by the red shade. (d) Quantification of selected sites, as indicated on top of each plot, in four brain regions. The y-axis denotes the log₂ intensity difference between U-50 and saline samples in the respective regions. (e) Immuno-histochemistry of ERK pT203/pY205. Selected images of stained hippocampus are shown on the top panel, with saline or U-50 treatment, and specific regions in hippocampus indicated within the images. CA3 = cornu ammonis field 3; DG = dentate gyrus. The bottom histogram shows quantification of immuno stain in various regions, including the abovementioned hippocampus. Ctx = cortex; CPu = caudate putamen, a subregion of striatum; Nac = nucleus accumbens, a subregion of striatum.

Fig. 3. KOR aversive signaling at synapses:

(a) Proteins assigned to different KEGG pathways, with particular phosphosites of each protein indicated by the position of the site in a circle. Empty, red, and blue circles indicate no change, increase and decrease of phosphorylation by U-50 stimulation, respectively. Significant U-50 altered sites are chosen from the pair-wise Welch's *t*-test between U-50 and saline samples, with cutoff of *p*-value < 0.05 and difference > 70% in each direction. Triangles next to the circles indicate changes of phosphorylation mediated by three different groups of KOR agonists: non-aversive, other aversive and RB64. The coloring is the same as above. (b) Immunohistochemistry in a cross-section of striatum using an antibody against DARPP-32 p97. (c) Quantification of selected phosphosites from (a).

Fig 4.

(a) Heatmap of the hierarchical clustering results of U-50 mediated PP2A substrates. These sites are de-phosphorylated after U-50 application with or without Tautomycin (Tauto) pretreatment (PP1 specific inhibitor), but they are not affected by the G-protein biased agonist 6'GNTI, and the PP2A inhibitors Fostriecin (Fost) or Calyculin (Caly) pretreatment combined with U-50 stimulation. The median intensity of three biological replicates in each condition was z-scored and colored as shown in the scale bar. **(b)** Selected sites from (a). The y-axis is the $\log_2$ difference between each condition and saline control.

Fig. 5. mTOR signaling pathway is involved in aversion, but does not increase of seizure threshold or diminish antinociception.

(a) Fischer's exact test on the group of sites that are significantly regulated by aversive agonists, but not by non-aversive biased agonists in striatum after 5 min of KOR activation.

(b) Same as (a) but in cortex after 30 min stimulation. The x-axis is the $-\log$ of p-value obtained from the Fischer's exact test, while the y-axis is the fold difference between the percentage of significantly differentially regulated sites that carried the depicted annotations over the percentage of all sites that carried the same annotation.

(c) Conditioned place aversion in mice (d) Seizure threshold in mice (e) Writhing test in mice. TEM stands for Temsirolimus, a mTOR inhibitor