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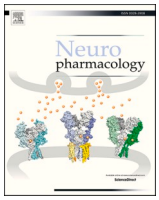

MSK1 is required for the experience- and ampakine-dependent enhancement of spatial reference memory and reversal learning and for the induction of Arc and BDNF

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ABSTRACT

There is considerable interest in the development of nootropics, pharmacological agents that can improve cognition across a range of both cognitive modalities and cognitive disabilities. One class of cognitive enhancers, the ampakines, has attracted particular attention by virtue of improving cognition associated with animal models of neurodevelopmental, neurodegenerative, and psychiatric conditions, as well as in age-related cognitive impairment. Ampakines elevate CNS levels of BDNF, and it is through this elevation that their beneficial actions are believed to occur. However, what transduces the elevation of BDNF into long-lasting cognitive enhancement is not known. We have previously shown that MSK1, by virtue of its ability to regulate gene transcription, converts the elevation of BDNF associated with environmental enrichment into molecular, synaptic, cognitive and genomic adaptations that underlie enrichment-induced enhanced synaptic plasticity and learning and memory, a property that MSK1 retains across the lifespan. To establish whether MSK1 similarly converts ampakine-induced elevations of BDNF into cognitive enhancement we tested an ampakine (CX929) in male WT mice and in male mice in which the kinase activity of MSK1 was inactivated. We found that MSK1 is required for the ampakine-dependent improvement in spatial reference memory and cognitive flexibility, and for the elevations of BDNF and the plasticity-related protein Arc associated with ampakines and experience. These observations implicate MSK1 as a key enabler of the beneficial effects of ampakines on cognitive function, and furthermore identify MSK1 as a hub for BDNF-elevating nootropic strategies.

1. Introduction

The pharmacological enhancement of cognition has been a much sought-after goal as it holds promise for the remediation of cognitive disabilities associated with a wide range of neurodevelopmental, neurodegenerative, psychiatric and age-related conditions. Among the various classes of cognitive enhancers, or nootropics, which includes histone deacetylase inhibitors (Burns and Gräff, 2021), cholinergic agents (Kamkwalala and Newhouse, 2017; Nguyen et al., 2022) and phosphodiesterase inhibitors (Heckman et al., 2018; Siegel et al., 2021), are the ampakines. Ampakines are positive allosteric modulators (PAMs) of the glutamate AMPA receptor (AMPAR), the development of which was pioneered by Lynch and colleagues in the early 1990s (Lynch, 1998). In those early studies ampakines were shown to enhance AMPAR-mediated currents and glutamatergic synaptic transmission in vitro and in vivo, and to improve performance in a number of

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behavioural tasks (Arai et al., 1994; Staubli et al., 1994). Further studies revealed that ampakines modulate AMPARs by slowing deactivation and desensitization kinetics. This extends the duration of glutamate-bound AMPAR openings and results in the observed enhancement of AMPAR-mediated synaptic transmission in the brain (Lynch and Gall, 2006). Subsequently, ampakines and other ampaPAMs have been tested in a wide variety of preclinical models and clinical settings for a range of neurodevelopmental, neurodegenerative, psychiatric and age-related conditions in which cognition is impaired, with many positive observations in animal studies, and encouraging results in human trials (Kadriu et al., 2021).

A key factor in the benefits observed in animal studies of ampakines is likely to be the elevation in brain-derived neurotrophic factor (BDNF) that has been observed in numerous studies at both the mRNA and protein level (Kadriu et al., 2021). These elevations in BDNF, particularly in the hippocampus, the subsequent activation of the BDNF TrkB receptor (Lauterborn et al., 2000, 2009; Jourdi et al., 2009; Cavalleri et al., 2018) and the initiation of intracellular MAPK signalling (Seese et al., 2020) ultimately likely lead to the enhancement of cognition. Indeed, elevations in BDNF and their importance for the benefits of cognitive enhancers have been reported for other classes of nootropics (Siegel et al., 2021; Nikita and Hannah, 2023) and may be a common feature of strategies designed to improve cognition.

An additional manipulation that elevates BDNF and which enhances synaptic plasticity and cognition is environmental enrichment (Rogers et al., 2019; Cooper and Frenguelli, 2021; Cutuli et al., 2022). Previous studies from our laboratory have demonstrated that the kinase activity of MSK1, a nuclear enzyme downstream of BDNF/TrkB signalling that regulates gene transcription via the phosphorylation of CREB and Histone H3 (Reyskens and Arthur, 2016), is necessary for the full genomic, molecular, synaptic, and behavioural benefits of environmental enrichment (Corrêa et al., 2012; Privitera et al., 2020; Cooper and Frenguelli, 2021; Morè et al., 2023). In particular, MSK1 is required for both spatial reference memory and cognitive flexibility (Privitera et al., 2020), a requirement that becomes more pronounced with age (Morè et al., 2023).

To test whether MSK1 also transduced the ampakine-mediated elevation of BDNF into enhanced cognition, we treated wild type (WT) mice and mice in which the kinase activity of MSK1 had been eliminated (kinase dead; MSK1 KD) (Corrêa et al., 2012) with an ampakine (CX929) and tested the mice for spatial working memory in the spatial alternation task, and for spatial reference memory and cognitive flexibility in the Morris water maze. In addition, we measured hippocampal BDNF and Arc/Arg3.1 levels after 7 and 16 days of CX929 treatment. We found that MSK1 is required for spatial reference memory and cognitive flexibility, and for the experience- and ampakine-dependent enhancement of BDNF. Similarly, we showed that Arc, a prime regulator of synaptic plasticity and cognition (Zhang and Bramham, 2021) and regulated by BDNF in an MSK1-dependent manner (Hunter et al., 2017), was also dependent upon MSK1 for its experience- and ampakine-driven expression in the hippocampus. These observations implicate MSK1 as a key mediator of the cognition-enhancing effects of BDNF in the mammalian brain, and moreover identifies the BDNF/TrkB/MSK1 pathway as a prime target for the development of cognitive enhancers for the remediation of a variety of developmental and acquired cognitive impairments.

2. Materials & Methods

2.1. Animals

The MSK1 KD mouse used in this study has been described previously (Corrêa et al., 2012; Daumas et al., 2017; Privitera et al., 2020; Morè et al., 2023). Briefly, Asp 194 in the endogenous MSK1 gene was mutated to Ala (D194A). This inactivates the N-terminal kinase domain of MSK1. Genotyping was conducted by PCR using the primers 5′-CGGCCATGTGGTGCTGACAGC-3′ and 5′-GGGTCAGAGGCCTGCA CTAGG-3′, which gives 378- and 529-bp products for WT and targeted alleles, respectively. All the mice used in this study were on a C57-Bl/6 J genetic background after at least four backcrosses from the original C57-Bl/6n strain used by Taconic Artemis to generate the mutant mice. Male WT C57-Bl/6 J mice purchased from Charles River UK were used for backcrossing with female MSK1 KD homozygous mutants.

Mice were maintained under a 12/12 h light/dark cycle (lights on at h 07.00 a.m.) in a facility kept at 20–24 °C and humidity 50–70%. All animal procedures conformed with local, national, and EU guidelines concerning the welfare of experimental animals, and the behavioural experiments were performed under the auspices of Home Office licence PPL 70/7821 granted to BGF. The mice have been deposited with the INFRAFRONTIER/EMMA repository at MRC Harwell, UK (https://www.infrafrontier.eu/emma/strain-search/straindetails/?q=13015).

2.2. Housing

All mice were housed in standard Tecniplast 500 individually-ventilated cages (IVC). Housing consisted of placing 2 to 4 male mice, weaned at P24 from a singly housed mother (from E14), into standard IVCs with a cardboard tube, sawdust and shredded paper as nesting materials, with food and water ad libitum.

2.3. Drug and dosing

CX929 (3,8-diethyl-2,3,7,8-tetrahydrobenzo [1,2-e:4,5-e']bis [1,3] oxazine-4,9-dione; Fig. 1A) was provided by Dr Julie Lauterborn (University of California, Irvine). On day 1 mice were given one single intraperitoneal (IP) injection (at h09:00) and on day 2 two IP injections (at h09:00 and h16:00) of CX929 (5 mg/kg in a 2 ml/kg volume of 10% (2-Hydroxypropyl)-β-cyclodextrin. From days 3 to day 12 mice were given a single IP injection (after behavioural testing was completed) of CX929 at 10 mg/kg (cyclodextrin 12.5%) and again on days 14 and 16. Days 13 and 15 were injection free. Control mice were instead administered equivalent volumes of 10% or 12% cyclodextrin. Two WT mice administered vehicle, 1 WT mouse treated with CX929 and 1 MSK1 KD mouse given CX929 had to be culled during the experiment due to unexpected weight loss, likely due to the vehicle.

2.4. Behavioural procedures

2.4.1. Testing outline

Male mice of both genotypes were between 3 and 5 months of age at the start of the treatment regime. Fig. 1B shows the experimental timeline. Spontaneous Alternation was run either on day 5 or 6 of CX929 or vehicle treatment. Habituation to the Morris Water Maze (MWM) occurred on days 7 and 8, with visual acuity testing with a visible cue placed on the platform taking place on days 9 and 10. The training phase of the MWM took place on days 11, 12 and 13, the MWM Reversal learning phase occurred on days 14 and 15 and the Probe test was conducted on day 16.

Brain tissue for western blotting was harvested on day 8 (3–4 animals per group) and on day 17 (4–5 animals per group) and stored at −80 °C for subsequent processing.

2.4.2. Neurological assessment

The assessment of neurological competency in WT and MSK1 KD mice was performed before the start of the behavioural testing and was adapted from the protocols described by Wolf and colleagues (Wolf et al., 1996). Mice were scored with 1 point for each neurological sign observed and 0 scores a fully healthy subject. All mice scored 0 points (data not shown).

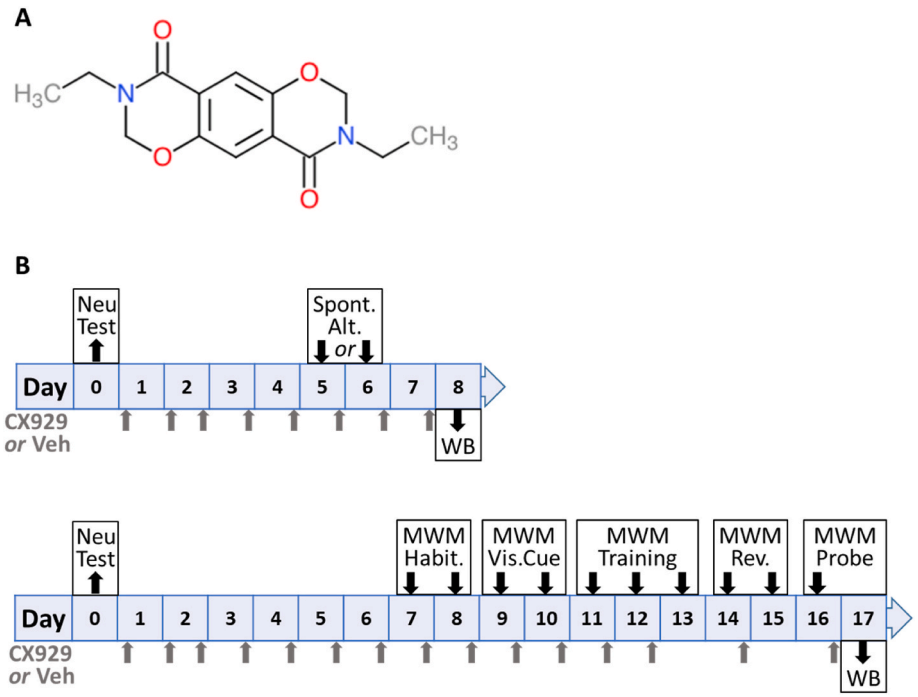


Fig. 1. A) Chemical structure of CX929. B) Schematic of experimental timeline. All animals were subjected to neurological testing (Neu Test; data not shown) prior to the start of the study. Arrows indicate when animals were dosed with either CX929 or vehicle (Veh). In one cohort of animals (10–12 animals per group; upper timeline), spontaneous alternation (Spont. Alt.) was tested on Day 5 or 6 with culling on Day 8 for western blotting (WB; 3–4 animals per group). In a separate cohort of animals (6–8 animals per group; lower timeline) dosing was the same, but with Morris water maze (MWM) testing for habituation (Habit.), visual acuity (Vis. Cue), training, reversal learning (Rev.) and the probe trial being conducted sequentially from Day 7 until culling for western blotting (WB; 4–5 animals per group) on Day 17.

2.4.3. Spontaneous alternation for hippocampus-dependent spatial working memory

A classical 8-radial arm maze for mice (Ugo Basile) was placed inside the empty water tank used for the water maze and only 4 out 8 arms (with walls) were kept open to form a cross. Each mouse was individually released in the centre of the maze and tracked for 10 min. The sequence of arm entries was scored. A correct alternation was considered to have occured when a mouse made one repetition in 5 entries. Two WT mice treated with CX929, 1 MSK1 KD mouse treated with CX929 and 1 WT mouse administered vehicle had to be excluded from the Spontaneous Alternation task because they showed arm bias (Oettinghaus et al., 2016; Pennucci et al., 2016).

2.4.4. Water maze reversal learning protocol for cognitive flexibility

Stage 1: Habituation: each mouse was placed on a 20-cm diameter platform located in the centre of a 180-cm diameter pool filled with opaque water (28 °C) and was allowed to observe the environment for 2 min. The pool was surrounded by curtains which did not allow the distal visual cues to be seen. Water level was ca. 1 cm above the top of the platform. Each mouse then received 3 consecutive trials (different starting points) where it was left free to swim in the pool for a maximum of 2 min and then placed on the platform and left there for 30 s.

Stage 2: Visual Cue: The platform was placed in the centre of the pool and a salient object was placed on it (yellow TV toy 6 × 6 × 5 cm). Each mouse received 4 consecutive trials (different cardinal starting points) where it was left free to swim in the pool for a maximum of 90 s. Water level was ca. 1 cm above the platform top. Water was kept at 26 °C. The pool was surrounded by curtains which did not allow the distal visual cues to be seen.

Stage 3a: Training: Curtains were removed. Water was kept at 26 °C. The platform was placed in the centre of the South-East quadrant or North-West (counterbalanced). Water level was ca. 1 cm above the platform top. Each mouse received 4 trials (ITI 120 s; different starting points) and was left free to swim in the pool for max 90 s and then left on the platform for 30 s.

Stage 3b: Reversal Learning: The platform was placed in the quadrant opposite to that used during Training.

Stage 4: 24 h delay Probe trial: No platform was present, and mice were given a single 90 s trial. Starting point was distal to the location of the platform during Reversal learning stage.

2.5. Western blotting

Mice were killed by cervical dislocation followed by exsanguination on either days 8 or 17. Hippocampi were dissected from surrounding brain tissues, homogenized in Eppendorf Scientific tubes with a pellet pestle in ice-cold solution composed of: 1 mM EDTA, 100 mM NaCl (pH 7.5), 1% Triton X-100, 1 mM sodium orthovanadate, 50 mM sodium fluoride, sodium pyrophosphate, 0.27 M sucrose, 20% NaN_3 and protease inhibitor cocktail (Roche). Homogenates were rotated for 1 h at 4 °C and centrifuged at 13,000 g for 15 min, the supernatant was collected, and protein concentration was determined using BCA protein assay (Pierce™ BCA protein assay kit # 23,225, Thermo Scientific™). Lysis buffer volumes were added to samples to equalise protein content between samples and 30 μg of protein per lane were separated in 14% SDS polyacrylamide gel electrophoresis system and transferred onto Hydrobond-ECL membrane (GE Healthcare) as previously described (Eales et al., 2014; Privitera et al., 2022). Membranes were blocked for 2 h with 5% BSA in PBS followed by incubation overnight at 4 °C with primary antibodies to BDNF (rabbit anti BDNF Sigma Aldrich # SAB2108004, 1:2000; 27 kDa), followed by goat anti-rabbit IgG-HRP H + L (Cell Signalling, 1:10,000) and blots imaged using Bio-Rad Gel Doc XR plus gel documentation system. The blots were then incubated with Arc/Arg3.1 (rabbit anti-Arc Synaptic Systems, # 156,003, 1:2500; 50 kDa) for 90 min at RT, followed by goat anti-rabbit IgG-HRP H + L (Cell Signalling, 1:10,000) and imaged. Blots were then incubated with mouse

anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Abcam, # ab8245, 1:5000; 37 kDa) followed goat anti-mouse IgG HRP LC (Jackson ImmunoResearch, 1:20,000). Primary and secondary antibodies were diluted in Tris-buffered saline Tween 20 (TBST) 0.5% BSA. Full images of all blots can be found in Supplementary Fig. 1.

2.6. Data analysis and statistics

The behavioural experiments were captured and analysed using AnyMaze 4.99 video tracking software. Western blots were imaged, and the signal intensity of the bands analysed, using the ChemiDoc™ MP Imaging System (Bio-Rad). Statistical analysis was performed using IBM SPSS 28. Two-way (genotype and drug treatment) M/ANOVAs or RM-ANOVAs (the within variable was the day of training in the MWM task) were performed as appropriate. Unless otherwise stated in the figure legend, the post-hoc analysis was performed with the Simple Main Effects via the Estimated Marginal Means with Sidak confidence interval adjustment. Statistical significance was taken to be $p < 0.05$, and exact p values are reported to three decimal places. Between-subjects variables were Genotype and CX929 treatment while the Within-subjects variable was the day of test in the water maze. Linear regression analysis of BDNF and Arc expression was performed using OriginPro 2021b. Data are presented as mean ± SEM and bar graphs display individual data points from the mice examined. All experiments were conducted blind to both genotype and drug treatment.

3. Results

3.1. The role of MSK1 in neurological function and the effects of ampakines on spatial working memory

We first established that the WT and MSK1 KD mice showed no neurological impairments at the start of the study that could confound the interpretation of the observations (Fig. 1B; Methods, data not shown). We detected no such impairments, consistent with observations made previously in young, middle aged and old WT and MSK1 KD mice (Privitera et al., 2020; Morè et al., 2023).

We began our investigation of the influence of CX929 and MSK1 on cognitive function by assessing spatial working memory in the Spontaneous Alternation task (Fig. 2A). In this task, mice have to sequentially visit four different arms of a four-arm maze before returning to a previously investigated arm. This establishes the animal's capacity to briefly store a record of its prior location over a 10 min time frame. We have shown previously that this task does not require MSK1 under standard housing conditions, where the performance of MSK1 KD mice is comparable to that of WT mice. However, under conditions of environmental enrichment when cognition is improved in WT mice, WT mice out-perform MSK1 KD mice, implying a requirement for the kinase activity of MSK1 in the enrichment-induced enhancement of cognition (Privitera et al., 2020; Morè et al., 2023).

In the Spontaneous Alternation task, both vehicle- and CX929-treated mice of both genotypes made a similar number of arm entries over the 10 min testing period (Fig. 2B). This indicates that neither genotype nor ampakine treatment influenced locomotor behaviour. In the assessment of spatial working memory (Fig. 2C) we observed no significant effect of CX929 treatment, but a mild trend towards a genotype effect on the percentage of correct alternations, with a similar weak trend favouring better performance in CX929-treated WT mice.

3.2. MSK1 is required for ampakine- and experience-dependent enhancement of spatial reference memory and cognitive flexibility

In the Morris water maze test for spatial reference memory, all groups learned the location of the hidden platform by the final day (T3) of training, with statistically better performance at T3 in the CX929-treated animals (Fig. 3A). Differences emerged during the two-day Reversal learning phase (Wolfer et al., 1998), during which WT mice performed better than MSK1 KD mice, consistent with previous observations (Privitera et al., 2020; Morè et al., 2023). CX929-enhanced performance was evident on R1, but this only persisted to R2 for CX929-treated WT mice, in contrast to CX929-treated MSK1 KD mice which did not improve on R2 (Fig. 3A). Heat maps of the cumulative location of the various groups on R2 show targeted searching by CX929-treated WT mice for platform location, but much less so for the other groups.

Probe trials taken 24 h later with the platform removed, showed more time spent in the target (Reversal) quadrant by WT mice, with significantly greater time spent in the quadrant by CX929-treated WT mice compared to CX929-treated MSK1 KD, and with cumulative heat maps of location confirming this observation (Fig. 3B). WT mice also performed better in latency to the previous Reversal platform location, with evidence of better performance in the CX929-treated WT mice (Fig. 3B). Thus, treatment with CX929 does not improve the cognitive deficit seen in mice lacking the kinase activity of MSK1 implying a role for this enzyme in the cognition-enhancing properties of ampakines.

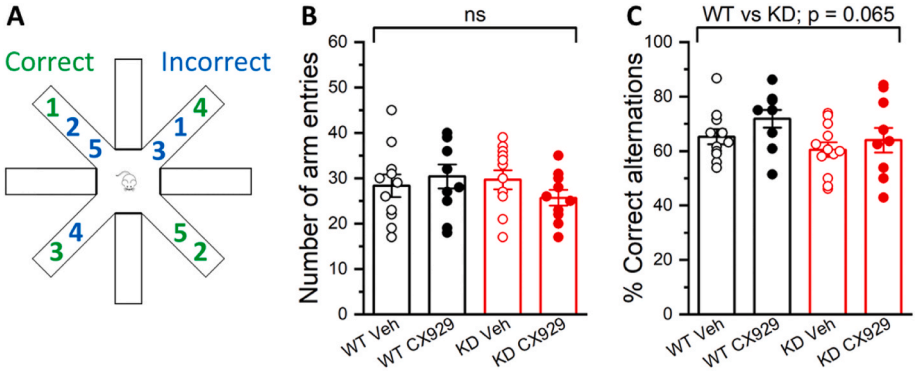


Fig. 2. Neither CX929 nor MSK1 influences spatial working memory performance. A) Visual depiction of the spontaneous alternation task illustrating one correct (green) and one incorrect (blue) alternation with the 1 repetition over 5 entries criterion. B) 5- or 6-day treatment with CX929 did not affect locomotor activity and spatial exploration in the spontaneous alternation task (Genotype effect: $F(1,39) = 0.56$, p: 0.459; Treatment effect: $F(1,39) = 0.18$, p: 0.673; Genotype x Treatment effect: $F(1,39) = 1.75$, p: 0.194). C) Treatment with CX929 fell short of significantly improving hippocampus-dependent spatial working memory in the spontaneous alternation task. There was a marginal effect for Genotype ($F(1,39) = 3.60$ p: 0.065) and a subsequent Simple Main Effects analysis showed a trend towards significance between WT and KD treated with CX929 ($F(1,39) = 3.02$, p: 0.090). WT (black bars and symbols) and MSK1 KD mice (KD; red bars and symbols) treated with vehicle (empty symbol) or with CX929 ampakine (solid symbol). ns; not significant. Bar graphs show mean ± SEM with data from individual animals superimposed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

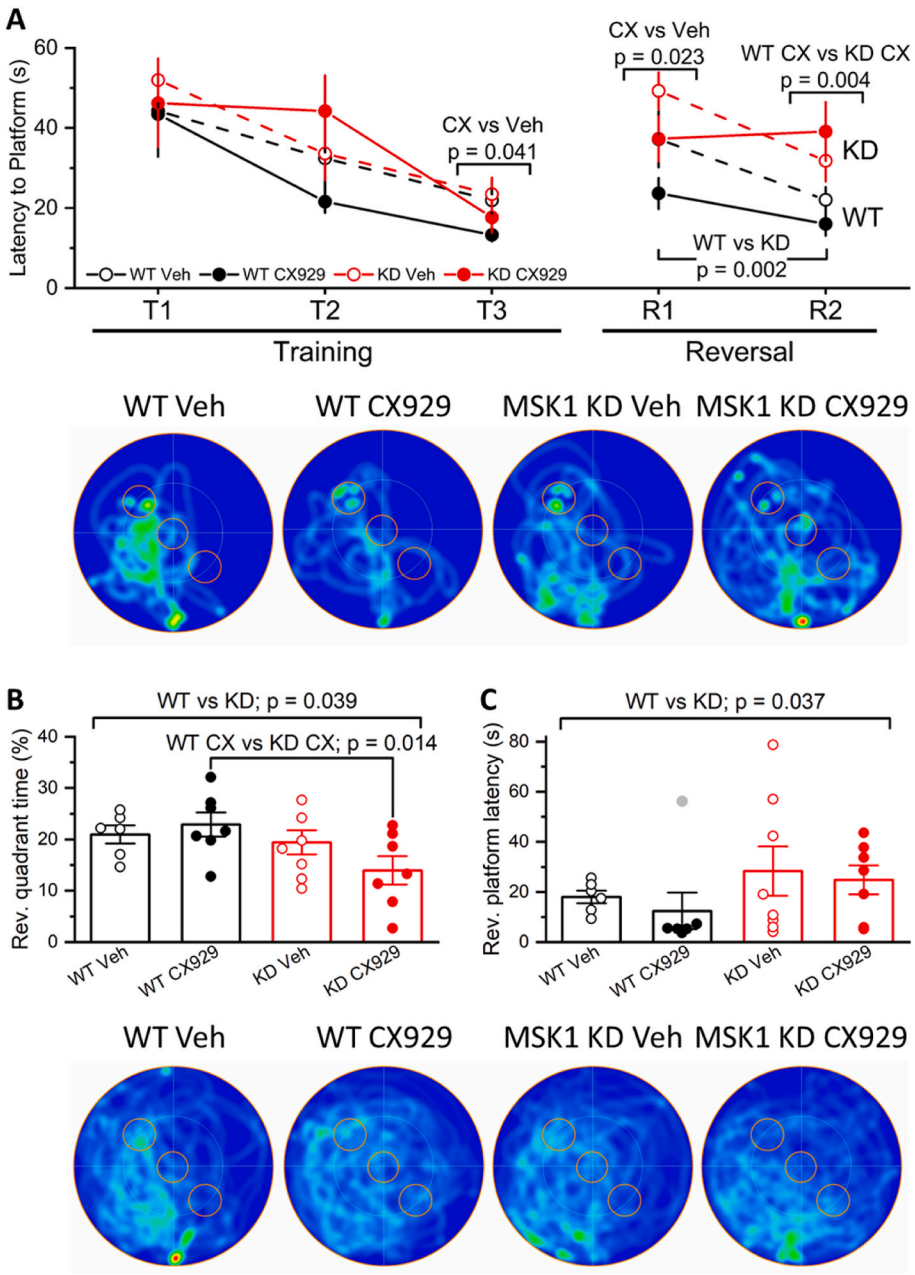
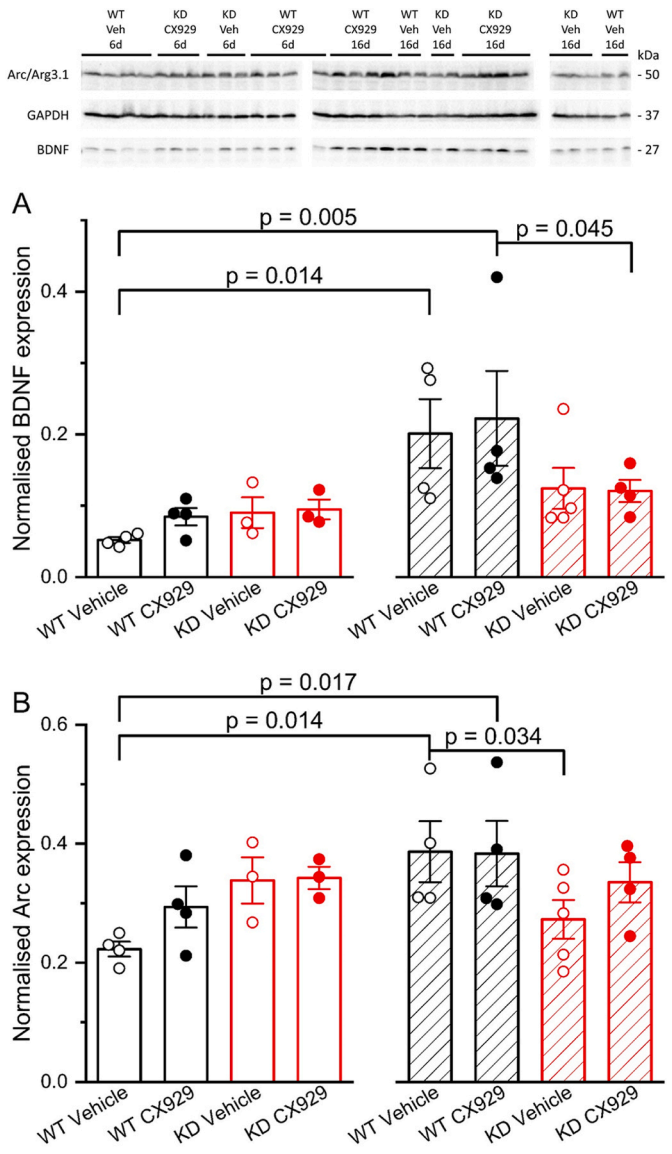


Fig. 3. MSK1 is required for CX929-dependent improvement of spatial reversal learning and reference memory. A) All mice improved over time in the Training (T) stage of this task F (2,48) = 16.65, p < 0.001. An analysis of the effects of CX929 on performance in the water maze showed that CX929-treated animals performed better at T3 (F (1,26) = 4.64, p: 0.041) and day one (R1) of the reversal learning phase (F (1,26) = 5.81, p: 0.023) than vehicle-treated mice. While the acquisition of the new (Reversal; R1-2) position of the hidden platform was enhanced in WT mice (Genotype F (1,24) = 11.78, p: 0.002) there was an effect of CX929 over these two sessions (CX929 x Session F (1,24) = 4.95, p: 0.036) that was not present in the MSK1 mutant mice. Indeed, the simple main effects analysis showed a significant difference between WT and MSK1 KD mice treated with CX929 (F (1,12) = 10.14, p: 0.004) on the second day of reversal learning. Heat maps show cumulative locations of the four groups on R2 of the Reversal learning phase (platform in the North-West quadrant). Data are shown as mean ± SEM. B) In the probe trial test of the water maze task CX929 treatment also significantly improved reference memory as shown by the time spent by WT mice in the quadrant where the platform was located during the reversal learning stage (Genotype F (1,24) = 4.78, p: 0.039). The Simple Main Effect revealed that within the mice treated with CX929 the difference between WT and KD was significantly different (F (1,24) = 7.03, p: 0.014) indicating a better performance of the CX929-treated WT mice. C) The WT mice also showed an overall significantly better performance measured as the latency to cross the 20 cm anulus where the escape platform was located in the Reversal stage (F (1,23) = 4.90, p: 0.037). One WT CX929 mouse was a significant outlier from among all WT mice (Grubb's Z = 2.832, critical value Z = 2.462 p: 0.005; shown as grey circle) and excluded from the analysis but included in the mean ± SEM of the bar chart. Heat maps depict cumulative location of the four groups during the Probe trial (previous platform location in the North-West quadrant). Bar graphs show mean ± SEM with data from individual animals superimposed.

3.3. MSK1 is required for the expression of plasticity-related proteins in response to nootropics and experience

To establish whether CX929 enhanced BDNF protein expression in the hippocampus of WT and MSK1 KD mutant mice, we extracted hippocampal protein for western blotting analyses from a cohort of four groups of mice exposed to a 7-day treatment, and a separate cohort of four groups of mice subjected to a 16-day treatment (Fig. 1B). CX929 treatment for 7 days, during which a brief (10 min) exposure to the Spontaneous Alternation task occurred two or three days earlier,



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resulted in an appreciable, but non-significant 63% increase in BDNF expression in CX929-treated mice (Fig. 4A). Of note was that in untreated and treated MSK1 KD mice, BDNF levels were higher than the levels seen in the untreated WT mice (by 74 and 82% respectively), and similar to those seen in the CX929-treated WT mice (Fig. 4A). This suggests a potential compensatory mechanism in mice lacking the kinase function of MSK1 to activate alternative BDNF-dependent signalling pathways through increased BDNF expression.

After 16 days of treatment with CX929 or vehicle, during which mice were also exposed to 10 days of training in the water maze (Fig. 1B), large (~4 fold) and significant increases in BDNF were observed at 16 days in WT mice, regardless of their exposure to CX929, compared to untreated WT mice at day 7 (Fig. 4A). This suggests that exposure to the water maze as a learning experience per se enhanced hippocampal BDNF. This does not exclude the possibility that achieving this elevated BDNF expression was accelerated by CX929 given the above-baseline values observed after 7-day exposure to the ampakine. In contrast, BDNF levels in the MSK1 KD mutant mice were comparable to those seen at Day 7, and significantly lower in CX929-treated MSK1 KD mice compared to CX929-treated WT mice. These data indicate that MSK1 is required for both the neuronal response to experience, as previously reported for environmental enrichment (Privitera et al., 2020; Morè et al., 2023) and to ampakine nootropics.

Fig. 4. MSK1, CX929 and experience influence BDNF and Arc expression. A) BDNF expression (27 kDA in blots above) normalized to GAPDH (37 kDa) across genotype (WT in black; MSK1 KD in red) and CX929 exposure (7 days, left open bars panels and filled symbols; 16 days, right hatched bars and filled symbols). There was a significant effect of Day; F (1,23) = 12.24, p: 0.002 and a significant interaction Genotype x Day; F (1,23) = 5.31, p: 0.031. The 63% increase in BDNF levels after 7 days of exposure to CX929 in WT mice increased significantly after training in the water maze and further exposure to CX929 (Day 16; one-tailed Dunnett's comparison with vehicle-treated WT mice as the control group p: 0.005) and significantly so above CX929-treated MSK1 KD mice at Day 16 (Simple Main Effect analysis; F (1,23) = 4.48, p: 0.045). Vehicle-treated WT mice showed an experience-dependent enhancement in BDNF levels between Day 7 and Day 16 (p: 0.014). BDNF expression in MSK1 KD mice, somewhat higher at baseline from control WT mice, did not change with CX929 or training. B) Arc expression (50 kDa in blots above) normalized to GAPDH (37 kDa) across genotype and CX929 exposure (labelling as per A) showed a Genotype × Day interaction (F (1,23) = 8.88, p: 0.007). There was a trend for an increase in the CX929-treated WT mice compared to vehicle-treated WT mice after 7 days of exposure, which became significant (Dunnett's comparison to vehicle-treated WT mice; p: 0.017) after 16 days of CX929 treatment and exposure to the water maze. Vehicle-treated WT mice responded to the testing in the water maze with a significant increase in Arc expression at Day 16 compared to 7 days of exposure (p: 0.014) and compared to untreated MSK1 KD mice (Simple Main Effect analysis; F (1,23) = 5.08, p: 0.034). Arc levels in the MSK1 KD mice, initially elevated above vehicle-treated WT mice, showed no change in response to water maze learning experience or CX929. Bar graphs show mean ± SEM with data from individual animals superimposed. Full western blots can be found in Supplemental Figs. 1A–C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To further probe the potential molecular mechanisms underpinning the actions of CX929, MSK1 and learning experience, we examined hippocampal protein levels of Arc from the samples that contributed the BDNF analysis. Similar to the observations made with BDNF, at Day 7, CX929-treated WT mice had higher (32%), but not significantly so, levels of Arc than vehicle-treated WT mice, while both vehicle- and CX929-treated MSK1 KD mice had similarly elevated levels above untreated WT mice (~50%; Fig. 4B). By Day 16, the early increase at Day 7 in CX929-treated WT mice had become significantly different from baseline values in untreated WT mice, while learning experience alone had elevated Arc expression in untreated WT mice (Fig. 4B). In contrast to these CX929-and experience-dependent increases in Arc expression in WT mice, no such changes were observed in MSK1 KD mice, in which untreated mice had significantly lower levels of Arc than untreated WT mice. This observation affirms the importance of MSK1 for Arc induction in response to experience.

Since BDNF can induce Arc expression in an MSK1-dependent manner (Hunter et al., 2017) we compared BDNF expression with that of Arc across genotype and treatment (Supplementary Fig. 2). We observed significant correlations between BDNF and Arc expression in vehicle- (linear regression analysis, Pearson's r = 0.724; p: 0.042) and CX929-treated (r = 0.892; p: 0.003) WT mice. MSK1 KD mice instead showed no significant correlations between BDNF and Arc after treatment with either vehicle (r = 0.355; p: 0.388) or CX929 (r = 0.745; p: 0.054).

In an attempt to better appreciate the relationship between ampakine, experience and the expression of the plasticity-related proteins BDNF and Arc, we considered exposure to short- or longer-term treatment with CX929 and acute or prolonged learning tasks as "doses" of cognitive stimulation. Accordingly, we plotted BDNF and Arc protein expression across the various experimental conditions relative to that seen in the 7-day vehicle-treated WT mice (Fig. 5). We observed a clear dose-response-relationship between experience and ampakine treatment in WT mice for both BDNF (Fig. 5A) and Arc (Fig. 5B). In contrast, in MSK1 KD mice the dynamic range for BDNF expression was greatly reduced (Fig. 5A), while for Arc expression the relationship between

experience and ampakine was absent. These observations suggest that the kinase activity of MSK1 is necessary for both the ampakine- and experience-driven enhancement of cognition, and for the expression of BDNF and Arc that likely contribute to this cognitive enhancement.

4. Discussion

We have used a mouse harbouring a knockin point mutation of the MSK1 gene, which eliminates the kinase activity of the enzyme, to examine the role of MSK1 in the widely reported cognition-enhancing properties of ampakines. The rationale for this approach is that the ampakines mediate their nootropic actions, at least in part, via the increased expression and release of BDNF, with the subsequent activation of TrkB receptors and the stimulation of the MAPK signalling pathway (Kadriu et al., 2021). MSK1 lies, within the nucleus, at the apex of this signalling cascade and transduces the activation of BDNF TrkB receptors and the MAPK pathway into the regulation of gene expression, primarily via the phosphorylation of CREB and Histone H3 (Arthur et al., 2004; Chwang et al., 2007; Remenyi et al., 2010; Hunter et al., 2017).

Previous instances of MSK1 critically regulating BDNF-dependent synaptic and cognitive process in the mammalian hippocampus includes homeostatic synaptic plasticity, as initially described by Rutherford and colleagues (Rutherford et al., 1998), in which the compensatory up- or down-regulation of synaptic transmission in response to synaptic activity deprivation or enhancement, respectively, was absent in hippocampal neurones from MSK1 KD mice (Corrêa et al., 2012). In addition, the environmental enrichment-induced genomic homeostasis, spinogenesis, expansion of the synaptic dynamic range, cognitive flexibility and persistence of memory were impaired or absent in MSK1 knockout (Karelina et al., 2012) or KD mice (Corrêa et al., 2012; Privitera et al., 2020; Morè et al., 2023). MSK1 thus represents a key hub for the integration and transmission of activity- and experience-dependent elevations of BDNF into genomic, structural and functional adaptations that support the enhanced cognition provoked by experience. Accordingly, it was apposite to ask whether MSK1 is recruited during ampakine-mediated elevations of BDNF, and whether it is responsible for some of the cognitive benefits reported for ampakines.

In this study we used CX929, a centrally-acting, short-lasting ampakine ($t_{1/2}$–15 min in rats) (Rex et al., 2005) that would be cleared from the body long before behavioural testing some 24 h after the previous dose. Importantly, CX929 has been shown to elevate BDNF (Rex et al., 2005; Kramar et al., 2012) and to activate the MAPK pathway (Seese et al., 2020) in WT animals in vivo in dosing schedules similar to the one used in this study.

CX929 did not affect locomotor activity during the 10 min trial in the Spontaneous Alternation task for spatial working memory, in keeping with observations made previously for CX929 in animals housed under standard conditions (Rex et al., 2005; Simmons et al., 2011; Baudry et al., 2012; Seese et al., 2020). In terms of influence on spatial working memory, there were no significant effects of either genotype or CX929 treatment, although there was a tendency for CX929-treated WT mice to perform better on this task. In the Delayed-Nonmatch-to-Sample test for working memory a different ampakine (CX516) did enhance performance in rats (Hampson et al., 1998), thus it is unclear why no such effects were observed in this study. One possibility is that the short (5 or 6 day) delivery of CX929 was not long enough for the benefits of the ampakine to develop. Indeed, this is consistent with the small effects on BDNF and Arc expression at this time (vide infra).

Performance on the water maze test for spatial reference memory was improved by the ampakine. While no differences were observed in the learning of platform location during the training trials between vehicle-treated WT and MSK1 KD mice, as shown previously (Daumas et al., 2017; Privitera et al., 2020; Morè et al., 2023), CX929 did improve performance on the third day of training regardless of genotype. These findings indicate a potential AMPAR-dependent, but MSK1-independent effect after repeated training, as was also seen in the first reversal day of the task.

Significant MSK1- and CX929-dependent differences in performance

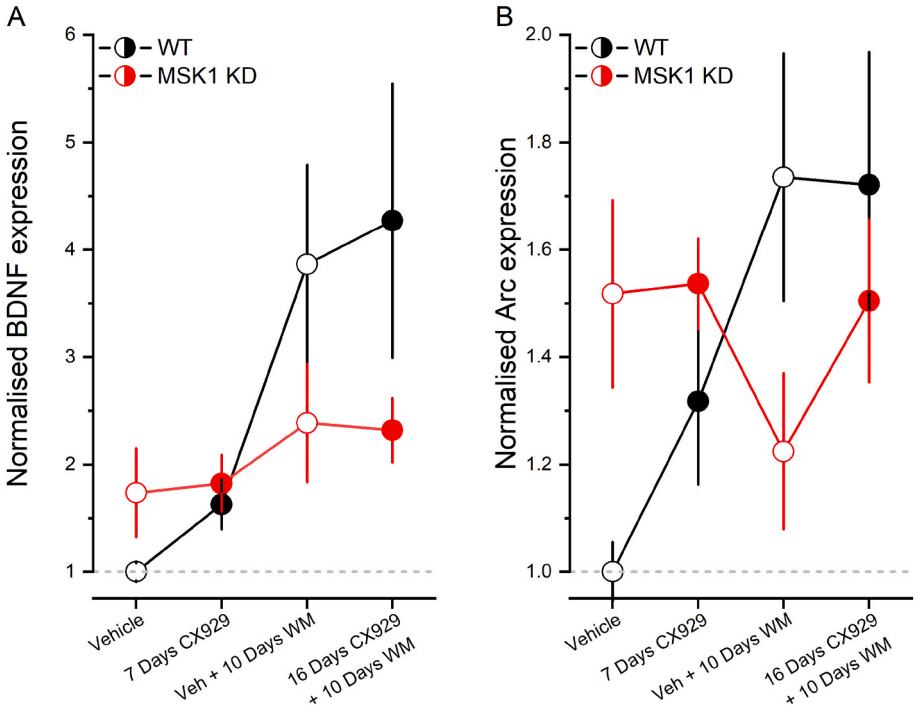

Fig. 5. Influence of MSK1 on BDNF and Arc expression as a function of experience and ampakine treatment. A) BDNF expression across treatments and genotypes relative to expression in 7-day vehicle-treated WT mice from Fig. 4A. BDNF expression showed an appreciable classic sigmoidal dose-response relationship in WT mice, whereas in MSK1 KD mice the range of expression was greatly reduced. B) Arc expression across treatments and genotypes relative to expression in 7-day vehicle-treated WT mice from Fig. 4B. In contrast to the strong dose-response relationship in WT mice, Arc expression in the MSK1 KD mice showed no relationship to experience or ampakine treatment. Data are expressed as mean ± SEM.

were revealed during the reversal learning phase in which WT mice performed better, as per previous studies (Privitera et al., 2020; Morè et al., 2023), but CX929-treated WT mice out-performed the CX929-treated MSK1 KD mutant mice. Similar superior performance by CX929-treated WT mice over CX929-treated MSK1 KD mice was observed during the Probe trial. These observations are consistent with the those made previously that enriched WT mice perform better than enriched MSK1 KD mice in these tasks (Privitera et al., 2020; Morè et al., 2023), and suggests that MSK1 is necessary to realise the full cognitive benefits of both enrichment and ampakines.

Remarkably, neither our previous intervention with environmental enrichment nor our current delivery of CX929 were capable of fully rescuing deficits in performance on the water maze in the MSK1 KD mice. This is in stark contrast to the improvements in cognitive and other deficits observed with either CX929 or environmental enrichment in experimental models of Huntington's disease (van Dellen et al., 2000; Simmons et al., 2009; Simmons et al., 2011; Novati et al., 2022), Angelman syndrome (Baudry et al., 2012; Jamal et al., 2017; Cosgrove et al., 2022), age-related cognitive decline (Frick et al., 2003; Lauterborn et al., 2016; Birch and Kelly Á, 2019; Morè et al., 2020) and Fragile X (Restivo et al., 2005; Lauterborn et al., 2015; Li et al., 2020; Seese et al., 2020). This implies that MSK1 is crucial in converting pharmacological- or experience-dependent elevations of BDNF into enhanced cognition for the remediation of the cognitive impairments associated with these conditions.

Direct measurements of hippocampal BDNF protein expression after 7 days or 16 days of treatment of WT and MSK1 KD mice with CX929 showed evidence of both ampakine- and experience-dependent elevations in WT, but not MSK1 KD mice. There was a trend towards increased BDNF expression after 7 days of treatment in WT mice, after which a large increase was observed after 16 days of treatment, which also included a 10-day period of training and testing on the water maze. Thus, while others have reported elevations in BDNF in response to CX929 and other ampakines per se, the timing of our sampling of BDNF, while suggestive at Day 7 and the inclusion of extensive training before Day 17, may not have been optimal to observe such an elevation between treated and untreated WT mice. It is possible that CX929 may have shifted the dose response curve for BDNF expression to the left allowing BDNF elevation to occur earlier during water maze training, and more rapidly achieving the same maximal response as that in untreated, but trained WT mice.

Accordingly, and likely due to the extensive exposure to the water maze, vehicle-treated WT mice displayed expression levels of BDNF similar to CX929-treated WT mice. Thus, CX929 treatment may have accelerated the increased expression of BDNF during a phase of water maze training that contributed to better consolidation of the task on T3 and during reversal learning. Importantly, in contrast to these CX929- and experience-dependent increases in BDNF expression seen in WT mice, no such increases were seen in MSK1 KD mice where BDNF expression, initially somewhat elevated over vehicle-treated WT mice, remained the same over the two observation periods, and was significantly less in CX929-treated MSK1 KD mice compared to CX929-treated WT mice. This reduced BDNF expression may have contributed to their inferior performance in the Reversal and Probe trial phases of the water maze test. Thus, it would seem that these two disparate stimuli (experience and CX929) both elevate hippocampal BDNF expression in an MSK1-dependent manner.

There was similar evidence for the requirement for MSK1 in the pharmacological and experience-dependent increase in Arc expression. As per BDNF, there was evidence of an early elevation in Arc expression by CX929 in WT mice after 7 days, with a further increase in Arc expression after 16 days of CX929 treatment. This early rise, similar to that seen with BDNF, may too have contributed to better and more consolidated acquisition of the water maze paradigm. Untreated WT mice at Day 16 (and after 10 days of water maze exposure) showed similar levels of Arc as seen in CX929-treated WT mice, and significantly greater Arc expression than untreated MSK1 KD mice, suggesting that learning experience per se contributes to Arc expression, an observation that has been made repeatedly in a wide range of learning tasks (Sauvage et al., 2019), and that MSK1 plays a pivotal role in experience-dependent Arc induction. Moreover, given the MSK1-dependence of Arc induction in response to BDNF (Hunter et al., 2017), the significant correlation between BDNF and Arc expression in WT mice, but absent in MSK1 KD mice, suggests a causal relationship between the two that requires in large part the kinase activity of MSK1.

That MSK1 was required for both the ampakine- and experience-dependent elevation in both of these key plasticity-related proteins was exemplified by the classic sigmoidal dose-response relationship between exposure to both CX929 and learning on the water maze that was observed in WT mice. This relationship was either greatly reduced in range (BDNF) or absent (Arc) in mice lacking the kinase activity of MSK1. Thus it would seem, consistent with other observations in homeostatic synaptic plasticity in vitro (Corrêa et al., 2012) and environmental enrichment in vivo (Corrêa et al., 2012; Privitera et al., 2020; Cooper and Frenguelli, 2021; Morè et al., 2023), that the kinase activity of MSK1 is necessary for the appropriate adaptation for both the nootropic effects of ampakines, and the cognition-enhancing effects of learning a spatial reference task, and the ability to display the cognitive flexibility required to successfully negotiate a reversal-learning derivative of the task.

This requirement for MSK1 for the ability to adapt in the face of prevailing synaptic, pharmacological and environmental activity may explain the discrepancy between the elevated levels of Arc seen in vehicle-treated MSK1 KD mice in this study, compared to MSK1 KD mice of the same sex and age seen in a previous study (Privitera et al., 2020). In that study, hippocampal Arc protein levels were measured in behaviourally and pharmacologically naïve WT and MSK1 KD mice. This contrasts with the situation in the present study in which control mice were subjected to seven days of intraperitoneal injection of vehicle. It is possible that, as a consequence of the injections, the vehicle treatment itself, and the handling associated with it, an MSK1-dependent adaptive response was evoked in the WT mice involving a downregulation of Arc that did not occur in the MSK1 KD mutants. Indeed, the ability to adapt to either the forced swim test or a variety of stress measures after chronic social defeat stress, in which total and phosphorylated hippocampal MSK1 (but not MSK2) were increased, was greatly impaired in MSK1/2 knockout mice (Chandramohan et al., 2008), and in mice with selective hippocampal viral knockdown of MSK1 (Ji et al., 2022), respectively. In fact, viral over-expression of MSK1 resulted in better adaptation to chronic social defeat stress and the maintenance of BDNF levels, TrkB phosphorylation and MAPK activation. This contrasts with MSK1 knockdown, which exacerbated the effects of chronic social defeat stress on BDNF-MAPK signalling (Ji et al., 2022). Thus, MSK1 seems also to be an integral part of the signalling cascade associated with adaptation to stressful conditions (Reul, 2014; Mifsud and Reul, 2018).

The novelty of our observations lies in revealing a previously unknown, but critical role for MSK1 in the interaction between ampakines and experience in enhancing BDNF and Arc expression and cognition. This aligns with our previous observations of the requirement for MSK1 in homeostatic plasticity, the full genomic, synaptic and cognitive response to environmental enrichment, and the observations of others of the need for MSK1 for the appropriate behavioural response to stress. These disparate pharmacological and experiential stimuli share a common feature in the BDNF-dependent signalling that they, and indeed other nootropics, initiate. Downstream of the BDNF-TrkB-MAPK pathway, MSK1 is well-placed to induce the gene expression changes that regulate the response to these stimuli to either adapt to stress or to benefit from the cognition-enhancing properties of nootropics and sensory experience. As such, MSK1 represents an important target for the development of novel therapeutic strategies designed to deal with affective or cognitive dysfunction.

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CRediT authorship contribution statement

Lorenzo Morè: Writing – review & editing, Writing – original draft, Visualization, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Lucia Privitera:** Writing – review & editing, Project administration, Methodology, Investigation. **Marcia Lopes:** Investigation. **J. Simon C. Arthur:** Writing – review & editing, Resources. **Julie C. Lauterborn:** Writing – review & editing, Resources. **Sonia A.L. Corrêa:** Writing – review & editing, Methodology, Funding acquisition. **Bruno G. Frenguelli:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

BGF is the Editor-in-Chief of Neuropharmacology.
No other authors have competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2024.110110.

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