Role of GluN2A containing NMDA receptors in memory reactivation: Molecular and behavioural evidence

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ABSTRACT. Whereas substantial evidence supports the role of the amygdala in fear-memory conditioning, little is known about the molecular mechanisms involved. It is currently believed that the MAPK-ERK signalling pathway may be key in supporting both consolidation and reconsolidation. Additionally, recent research has suggested that GluN2A NMDA receptors of the basal lateral amygdala are required for memory restabilisation, although their functions in memory reactivation are unclear. We predicted that GluN2A NMDARs in the basal lateral amygdala (BLA) would be involved in mediating memory reactivation through the MAPK-ERK signalling pathway. To test this hypothesis, we used Western-blot to analyse subcellular localization of ERK 20 minutes after fear-memory reactivation in rats treated with a GluN2A antagonist directly in the BLA (NVP-AAM0077). Nuclear ERK concentration was not found to be significantly modified post reactivation in NVP-treated animals relative to control groups (vehicle). Nevertheless, a trend was found in the cytoplasm, suggesting that GluN2A NMDARs may contribute to short-term adaptation of the ERK signalling during reactivation. Together, these findings provide insights for future research. Alternative research techniques and methodological approaches are considered.

1. Introduction

The field of memory research has long been dominated by memory consolidation theory, according to which memories are stored and stabilised over time after a new learning experience (McGaugh, 2000). In 1968, Misanin & Lewis were the first to show that post-retrieval memory could be disrupted (Misanin & Lewis, 1968). The evidence for a labile phase of memory during reactivation triggered further research on the mechanisms underlying post-reactivation storage, a process that has come to be known as reconsolidation (Karim Nader, 2003). Over the past ten years, an increasing number of research papers have focused on the cellular processes involved in reconsolidation.

Capitalizing on decades of research in fear memory formation, the role of protein synthesis has been thoroughly investigated in the amygdala and hippocampus – two brain regions known to be involved in the formation and reactivation of fear memories (LaBar & Cabeza, 2006). Using an auditory fear-conditioning paradigm, Duvarci et al. (2005) showed that infusing the inhibitor of protein synthesis anysomycin in the lateral amygdala following reactivation of a fearful memory led to amnesia. These results were consistent with the hypothesis that reconsolidation in the lateral amygdala requires protein synthesis (Nader et al., 2000).

It is well established that in addition to protein synthesis, consolidation requires the activation of various signalling pathways, among which the extracellular signal regulated kinase mitogen activated protein kinase (ERK-MAPK) plays a key role in contextual and cued fear learning (Sananbenesi, 2002; Trifilieff et al., 2007). The ERK-MAPK pathway is a cytoplasm-to-nucleus module, in which Raf proteins phosphorylate MAPKKs (MEK), which in turn activate ERKs. Once activated, ERKs translocate to the nucleus where they regulate transcription factors (Peyssonnaux & Eychène, 2001). Hence, the presence of ERKs in the nucleus suggests ERKs activation downstream the ERK-MAPK signalling pathway.

Evidence for the role played by the ERK-MAPK pathway in consolidation and reconsolidation of fear memories is strong. In rodents, MEK inhibitors pre-

venting ERK activation have been shown to disrupt cued fear memory consolidation in the amygdala (Schafe et al., 2000). Furthermore, Duvarci et al. (2005) have found that the infusion of MAPK kinase inhibitor in the lateral amygdala during memory reactivation also blocked reconsolidation, thus confirming the involvement of the ERK-MAPK pathway. Together, these findings provide strong evidence that ERK-MAPK signalling is a crucial component of fear-memory reconsolidation in the amygdala, although the upstream activators of ERK have not been well established.

ERK-MAPK activity has been linked to the NMDA subtype of glutamate receptors (NMDAR) of the amygdala, which have been shown to play a critical role in reconsolidation of fear-conditioned memories (Lee et al., 2013; Pedreira et al., 2002). NMDAR are commonly made of three major subtypes, GluN1/2/3, arranged using four subunits (GluN1-3A-D) (Dingledine et al., 1999). NMDARs of the basal lateral amygdala appear to mediate reconsolidation through molecular signalling via the GluN2A subunit (Milton et al., 2013). The contribution of amygdala GluN2A receptors to the formation of fear memory has previously been investigated in Pavlovian conditioning paradigms using infusions of NVP-AAM-077 (NVP), a GluN2A antagonist (Dix et al., 2010; Weitlauf et al., 2005). However, the cellular mechanisms underlying the molecular activation of GluN2A during reactivation have not yet been investigated.

We explored the effects of a GluN2A NMDAR antagonism, using NVP, in the basolateral amygdala specifically prior to cued-fear memory reactivation. Upon activation, ERK has been shown to translocate from cytoplasm to nucleus, where it modulates DNA transcription either through direct phosphorylating transcription factors or through intermediate substrates kinases (Davie & Spencer, 2001; Murphy et al., 2002). ERK also has a number of cytoplasmic targets, which can mediate short-term adaptations of the signalling cascade (Wortzel & Seger, 2011).

Based on previous research suggesting that GluN2A NMDAR may play a crucial role in fear memory consolidation and the known connection between NMDAR and the MAPK-ERK signalling pathway, we hypothesized that the activation of ERK in subcellular compartments would be reduced subsequent to NVP infusions, confirming the implication of the MAPK-ERK signalling cascade in the GluN2A NMDAR in memory reactivation. To test this hypothesis, we fractionated tissues in the basal lateral amygdala (BLA) and we used SBS-polyacrylamide gel electrophoresis (Western-Blot) as a primary method to detect the phosphorylated ERK (pERK) in the nucleus and cytoplasm. We used densitometry to assess differences in ERK activation post-reactivation between NVP (NVPRet) and vehicle treated rats (VehRet) and compared the results with a control group of NVP (NVPNoRet) and vehicle treated rats (VehNoRet) in a no-retrieval condition.

Western-Blotting has been shown to be particularly efficient in isolating and quantifying specific proteins (Towbin et al., 1979). The ability to separate proteins by sizes through electrophoresis and to detect pERK with targeted antibodies after electro-transfer was a key element in choosing this method. By evaluating the presence of ERK in our different treatment groups, we could compare the trafficking of pERK between cytoplasm and nucleus. In this report, the Western-Blot method is thoroughly discussed in the context of our experiment. Limitations are considered and alternative methodological approaches are explored.

Overview of the experiment

Personal responsibilities: Behavioural assessment of Pavlovian conditioning, Cryostat sectioning, fractionation of BLA tissues, protein extraction, Bradford protein assay, electrophoresis, densitometry and statistical quantification.

2. Methods and Material

Subjects

The subjects consisted of 24 male Lister-Hooded rats weighting 250-300g (Charles River Laboratories) housed in pairs on a reversed light-dark cycle. Subjects were food restricted but not deprived (25g per rat per day). All procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986.

Surgery

Subjects were implanted a stainless steel guide cannula in the basolateral amygdala nuclei of each hemisphere for intra-cerebral drug administration, as described by Milton et al. (2008). Obstructers were inserted in the cannulae at all times to main-

tain integrity between infusions. A 7-day recovery period was observed prior to starting testing and behavioural scoring. The coordinates were 3.6 mm posterior to bregma, 4.5 mm lateral to the mid-line, and 3.6 mm ventral to dura mater.

Intra-cerebral infusions

Rats were infused solutions intra-cerebrally using a syringe pump (28 gauge plastic, projecting 2mm beyond the guide cannula) at a rate of 0.25 µl min for a period of two minutes. The syringe was removed one minute after the infusion ended to ensure adequate diffusion of the solution. All rats received a habituation to infusion at the end of the first training session, consisting of a 0.5µl sterile saline solution administered evenly through each cannula. Prior to the last reactivation session, half the rats were administered a pure solution of phosphate buffered saline (PBS). The other half received a GluN2A NMDAR antagonist, NVP. The solution consisted of a concentration of 5 µg/ml NVP per litre in PBS and was injected with the same protocol used during habituation.

Behavioural procedure

Behavioural testing took place in four conditioning chambers (Paul Frey). Rats first underwent a 1h habituation period over 2 days in one of the chamber during which no auditory stimuli were presented and no electrical shock administered. The first set of fear conditioning session took place 24h later in the same experimental chamber. Rats were given 30 minutes to habituate, subsequent to which the first CS (clicker sound 10 Hz, 80 dB, 60s) was triggered, immediately followed by a 0.5 mA, 0.5-second electrical foot shock (US) delivered through the ground grid. This combination of clicker CS-US pairing was repeated 3 times with an intertrial of 5 minutes between the last shock and the next CS.

A memory reactivation test was conducted 24h later, where rats were exposed to a 4 minutes context exposure followed by the CS. After another 24h period, the rats were divided into four groups. A group of 12 rats was infused the same saline solution tested on the training day whereas another group of 12 rats was infused the NVP solution. The same memory reactivation test was performed on half the subjects of each set (6 rats infused with saline and 6 infused with NVP). The other half was not tested subsequent to the injection to be used as a control group. Data for the training and reactivation sessions was recorded via CCTV on a DVD for subsequent manual scoring of behaviour.

Data analysis

The behavioural data recorded was scored individually by three observers blind to treatment and results were compared to ensure reproducibility. Scores were attributed manually with 1 corresponding to freezing (inertia apart from breathing) and 0 corresponding to movement. The percentage of time freezing was computed for 1-minute pre-CS and during the CS at 5 seconds intervals. The results were compared with a baseline percentage of freezing for each rat prior to any fear conditioning pre-CS. Statistical analyses were performed with SPSS v.21 for Mac using a mixed-model ANOVA. Freezing over time (Pre CS, CS1, CS2 and CS3) was used as within subject factors with four repeated measures. Treatment (Vehicle or NVP) and retrieval groups (Ret or NoRet) were used as between-subject factors. Deviations from sphericity were identified with the Mauchly's sphericity test and corrected using Greenhouse-Geisser equation if < 0.75 and Huyn-Feldt equation if >0.75 (Cardinal & Aitken, 2013).

Histology

Rats were sacrificed by carbon dioxide asphyxiation approximately 20 minutes after the last session, which is the expected duration for ERK activation (Merlo, 2014). Brains were rapidly frozen at -80 °C. Brain sectioning was performed at -17 degrees Celsius using cryostat at 150 µm and 30 µm samples were regularly taken through the BLA to be preserved in PFA and stained with violet cresyl. The position of the cannulae within the amygdala were observed and recorded on a rodent brain atlas (Paxinos & Franklin, 1997) for each rat during sectioning. The 30 µm stained samples were used to verify the placement of cannulae in the amygdala using light microscopy. Amygdala tissues were extracted from the 150mm samples using a 0.99 mm diameter puncher and stored at -80 degrees Celsius for subsequent West-

Protein extraction

Cytoplasmic protein extracts and nucleic protein extracts were obtained by individually homog-

enizing the BLA tissue from each animal in a glass-glass Weathon dounce with 100 µl of buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, 1 g/ml Pepstatin A, 10 µg/ml leupeptin, 0.5 mM PMSF, and 10 µg/ml aprotinin) centrifuged at 1000g for 15 min at 4°C. The protein extracts (supernatant) were subsequently stored at -80°C and resuspended twice with a 20 minutes interval in 25 µl of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.84 mM KCl, 0.5 mM DTT, 0.5g mM EDTA, 1 µg/ml Pepstatin A, 10 µg/ml leupeptin, 0.5 mM PMSF, and 10 µg/ml aprotinin, 50% v/v Glycerol). The pellet was then centrifuged at 11,000g for 15 minutes at 4°C. The protein content was identified by Bradford assay.is the expected duration for ERK activation (Merlo, 2014). Brains were rapidly frozen at -80 °C. Brain sectioning was performed at -17 degrees Celsius using cryostat at 150 µm and 30 µm samples were regularly taken through the BLA to be preserved in PFA and stained with violet cresyl. The position of the cannulae within the amygdala were observed and recorded on a rodent brain atlas (Paxinos & Franklin, 1997) for each rat during sectioning. The 30 µm stained samples were used to verify the placement of cannulae in the amygdala using light microscopy. Amygdala tissues were extracted from the 150mm samples using a 0.99 mm diameter puncher and stored at -80 degrees Celsius for subsequent Western-Blotting.

Western-Blotting

Cytoplasmic and nucleic protein samples were prepared with 2x Laemmli and Lysis buffers boiled for 5 minutes at 100°C. Cytoplasmic and nucleic proteins were separated using a 10% SDS-PAGE and transferred into nitrocellulose membranes using Transblot system set at 150V for 45 minutes. The transfers and well positions were verified with Ponceau Rouge. Membranes were blocked in 0.1% condensed milk Tris-Buffered-Saline (TBS), 0.1% Tween20 and subsequently rinsed 3 times with TBS to remove non-significant proteins. Blots were incubated overnight with Phospho-p44/42 MAPK (Erk1/2; Thr202/Tyr204; 1:500; Cell Signaling Technology) and mouse anti-actin (AC-15; 1:5000, AbCam); in blocking solution and rinsed 3 times with TBS-T.

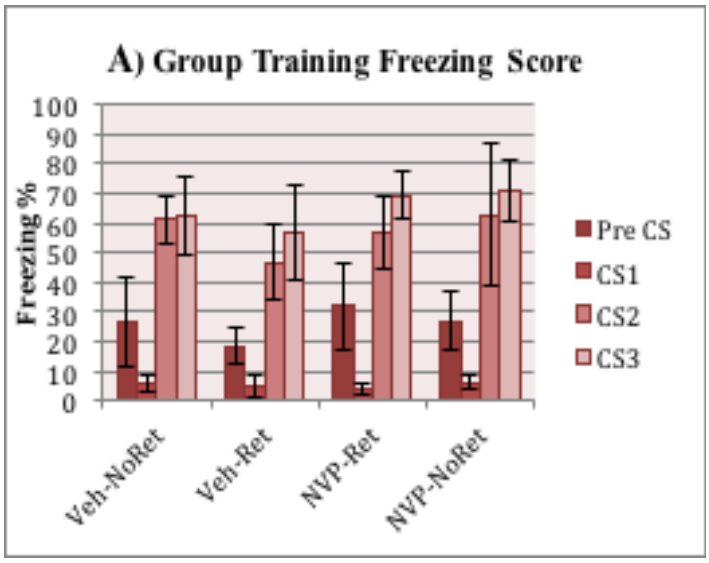
Membrane surfaces were coated with chemiluminescent reagents (GE Healthcare) and placed in a cooled CCD camera (ChemiDoc-It, UVP). 8 Images were taken over 40 minutes using VisionWorks. Captures were analysed and quantified with ImageJ software (version 1.48v, National Institutes of Health). Mouse pERK antibody working concentration was controlled to ensure a linear relationship between the protein content loaded in the blot and the intensity of signal revealed. The variations in protein load were controlled by using actin as a normalizing criterion for each sample.

Measures of the optical density of the bands were taken and optical density of the experimental group was calculated. Values were normalized to the mean optical density of the control group. Each sample was run in duplicate. Analysis of the four groups was performed with a one-way ANOVA using Dunnet's test for comparison with control group VehNoRet. Unpaired T-tests were used when a comparison between two groups was required.

3. Results

Behavioural data: GluN2A-NMDARs are not required for memory reactivation

All four groups of rats were conditioned by pairing a clicker sound (CS) with an aversive electric foot shock. As expected in Pavlovian fear conditioning paradigms, all rats showed a marked increase in the percentage of freezing after the first pairing with no significant variability between groups ($F_{3,15} = 35.4$, $p < 0.001$, $\eta^2 = 0.87$). The following day, each rat was presented with one CS and all four groups showed strong fear memory retention (Fig.1), as demonstrated by a large increase in freezing time rate after hearing the auditory CS ($F_{1,17} = 242.5$, $p < 0.001$, $\eta^2 = 0.93$).



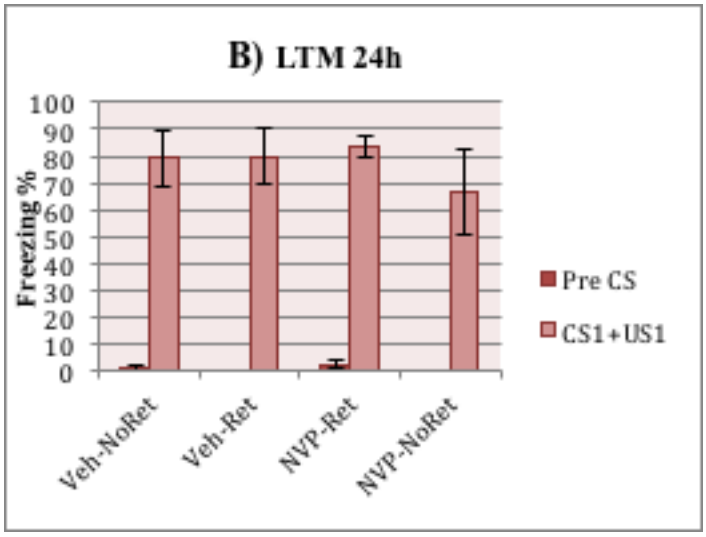


Figure 1. Pavlovian conditioning and reactivation session - Height of bar = mean, Error bar = ± 1 SEM
A) All groups show conditioned response to CS after first CS+US pairing with no significant variations. B) Conditioning is stable 24h after the training session, as demonstrated by a sharp increase in freezing rate after hearing the CS in the reactivation session.

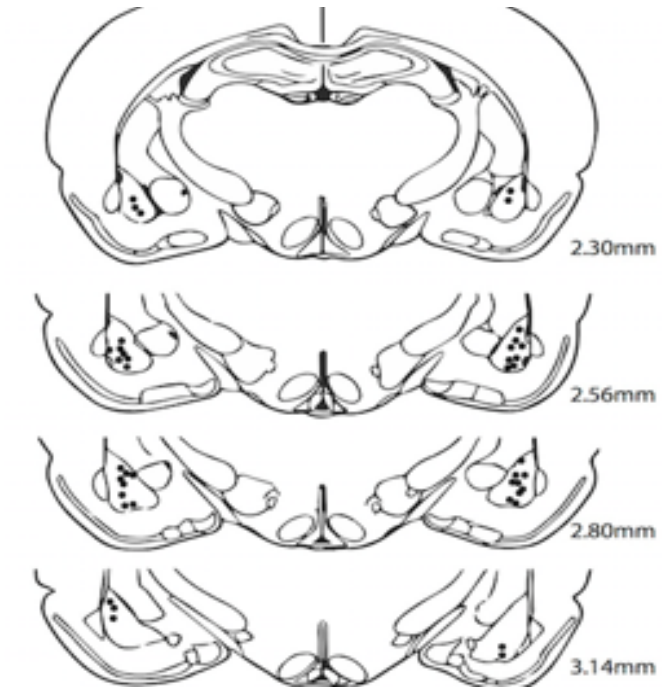


Figure 2. Cannulae placements. Cannulae placements were within the BLA for 21 rats. 3 rats were taken out of the sample due to misplaced cannula. This schematic representation of the brain adapted from Paxinos & Watson (2004) shows the sites for both Veh and NVP groups (black circles). Coordinates are given from bregma.

Half of the subjects were tested 24h later (Ret Group). A vehicle solution (n=5) or GluN2A-NMDAR antagonist NVP (n=6) were infused in the BLA of the tested group (Fig.2) prior to a further exposure to the CS. Freezing rates were measured Pre CS and post CS for each subgroup.

For both groups, fear conditioning remained stable with a sharp increase in freezing rate post CS ($F_{1,9}$ =66.94, $p < 0.001$, η^2 =0.09). Consistent with previous findings (Milton et al., 2013), infusion of NVP prior to the memory reactivation session did not significantly affect freezing response to CS in the treated group ($F_{1,9}$ =3.38 p =0.099 η^2 =0.273).

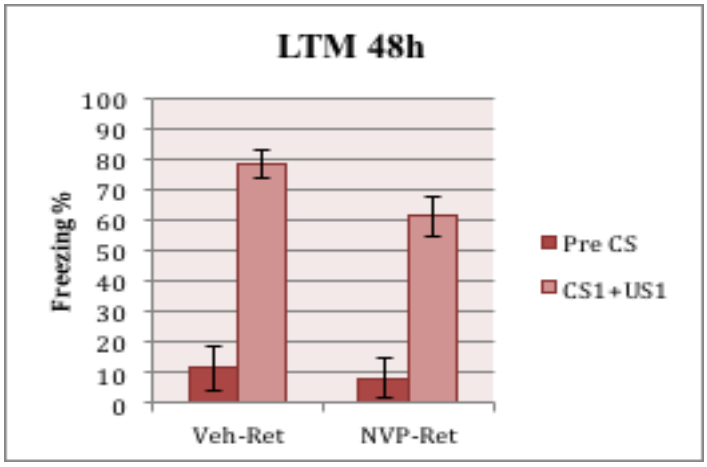


Figure 3. Freezing rate in reaction to CS does not decrease significantly after infusion of NVP vs. infusion of control vehicle solution.

Hence, NVP did not have an acute effect on reactivation of fear-conditioned memory during the test at 48h. These results suggest that GluN2A NMDARs are not required for reactivation.

Molecular data I: Blockade of GluN2A does not alter ERK signalling in the nucleus

Quantification of ERK activation in the nucleus with densitometry did not reveal significant statistical differences between the four groups, $F_{3,14}$= 0.447, p= 0.723 (Fig.4). Most importantly, GluN2A blockade with NVP did not appear to significantly reduce nuclear ERK activation relative to the VehRet group post reactivation, t_8= 2.06, p= 0.073. These results suggest that NVP-blockade of GluN2A does not affect the activation of ERK in the nucleus.

The expression of ERK in the nucleus was lower for the Veh group in the retrieval condition than in the no-retrieval condition, t_8= -7.349, $p < 0.001$; con-

versely, ERK was slightly more activated in the nucleus of the NVP group in the retrieval condition than in the no-retrieval condition, although the difference was non-significant, t6= 0.507, p= 0.630. Thus, a clear relationship between pERK in the nucleus and memory reactivation cannot be established. It is important to note that expression of ERK in the nucleus of the VehNoRet group was subject to a large variability (SEM = 40.17).

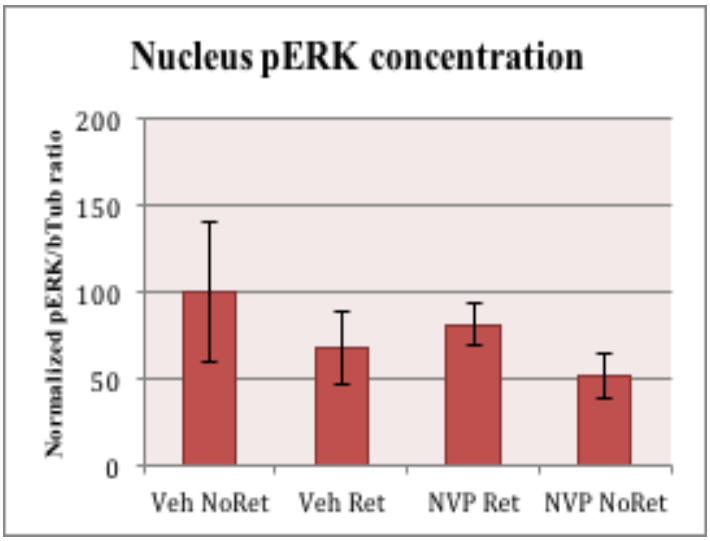


Figure 4. pERK concentration in the nucleus is not significantly decreased in the NVPRet group relative to the three control groups.

Molecular data II: GluN2A might play a role in mediating short-term adaptations of ERK signalling

Relative to the VehRet group, a reduction in ERK activation was observed in the cytoplasm of the NVPRet group post reactivation, although the difference was non-significant, t8 = -0.380, p = 0.714. No significant difference in ERK concentration was observed in the no-retrieval condition between the NVP group and the vehicle group, t8= 0.962, p= 0.364. Nevertheless, the results suggest a trend whereby ERK activation is increased in VehRet relative to VehNoRet. Conversely, GluN2A blockade appears to reduce ERK activation in the NVPRet relative to the NVPNoRet control group (Fig.5). Together, these findings indicate that GluN2A NMDAs might play a key role in mediating short-term adaptations of ERK signalling. Testing on a larger sample is necessary to establish whether the differences are statistically significant.

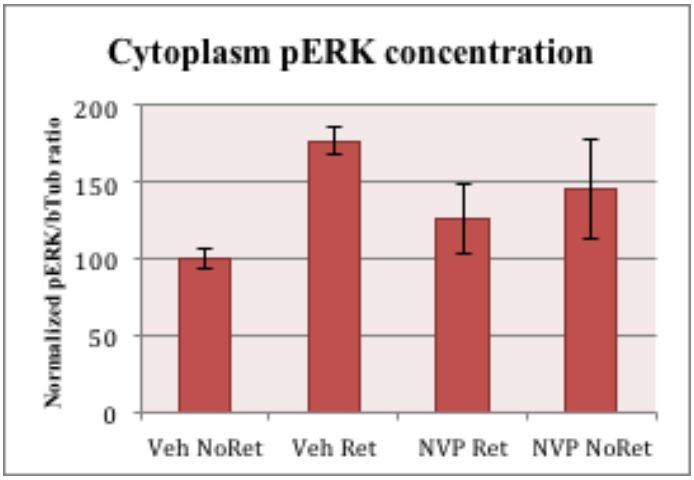


Figure 5. pERK appears reduced in the cytoplasm for the NVPRet group relative to the NVPNoRet group. Conversely, pERK appears increased in the VehRet group relative to the VehNoRet group. In addition, pERK is lower in the NVPRet group than in the VehRet group.

4. Discussion

Role of GluN2A NMDARs in memory reactivation

The lack of a significant difference in freezing rates between NVP-treated and vehicle-treated animals during the reactivation session suggests that GluN2A NMDA receptors do not play a key role in regulating fear-conditioned behaviour. These results confirm previous reports reporting that GluN2A are required for restabilisation but not for destabilisation of fear memories (Milton et al., 2013). As our experiment sought to investigate the molecular mechanisms involved in restabilisation, rats were to be sacrificed 20 minutes post-reactivation to analyse subcellular ERK activation. The chosen timeline was based on a protocol designed by Merlo et al. (2014) to explore changes in pERK during the restabilisation phase. Nevertheless, it is important to note that the process of restabilisation could be initiated or reach a peak after a longer delay. Accordingly, it is possible that ERK activation would reveal different patterns if rats were sacrificed after 60 minutes (Merlo et al., 2014).

Although GluN2A NMDARs do not appear to mediate behavioural response to fear memory reactivation, GluN2B antagonists have been shown to significantly impact fear memories in rats (Milton et al., 2013). From this observation, it has been suggested that GluN2B NMDARs are required for destabilisation of fear memories. In consequence, our experiment de-

sign could be adapted using a GluN2B antagonist before reactivation to prevent anisomycin-induced amnesia. In this case, we would predict that freezing rate would be subsequently reduced post-reactivation in rats treated with the GluN2B antagonist. The molecular mechanisms involved could be investigated with Western-Blot.

Molecular aspects of the ERK signalling pathway in memory reactivation

Our experiment could not establish a link between memory reactivation and downstream nuclear ERK signalling. Although no significant differences were found in cytoplasmic ERK activation, a trend was observed whereby GluN2A blockade resulted in reduced ERK activation in the NVPRet group relative to the NVPNoRet group. Conversely, activation of ERK was increased in the VehRet group relative to the VehNoRet group. Considering the sample size, the possibility of this inversed relationship being significant cannot be excluded. Indeed, as suggested by our results, it is not unlikely that reduction in cytoplasmic ERK activation resulting from GluN2A blockade would be statistically significant in a larger sample.

Although translocation of ERK in the nucleus plays a key role in DNA transcription, ERK has been shown to regulate gene expression through indirect mechanisms involving cytoplasmic targets (MacDonald, 2004). For example, one target of ERK is the cytoplasmic MAPK-activated protein kinase 2 (MAPKAPK2). Evidence suggests that MAPKAPK2 may be involved in stabilising actin filaments during osmotic stress, which contribute to the regulation of both transcription and gene expression (MacDonald, 2004; Zheng et al., 2009). Similarly, ERK regulates transcription through phosphorylation of Ribomosal protein S6 Kinases (RSKs). RSKs appear to modulate nuclear Fos expression and CREB phosphorylation, both of which have been shown to accompany memory retrieval and are likely to play a key role in memory reconsolidation (Hall et al., 2001).

ERK also appears to phosphorylate multiple cytoplasmic substrates and to activate several other kinases. For instance, ERK seems to induce phosphorylation of ETS domain-containing protein Elk-1, which may be involved in relaying glutamatergic signals to the nucleus (Davis et al., 2000). In addition, ERK-induced Elk1 phosphorylation has been reported to force Elk1 translocation to the nucleus, where it is likely involved with synaptic plasticity and learning (Besnard et al., 2011).

Furthermore, several cytoplasmic components are implicated in ERK negative feedback regulation, which allows signals to be terminated. One of the ways ERK suppresses signaling is by phosphorylating Son of Sevenless (SOS), which interferes with its binding to the Grb2 domain, hence inhibiting Ras activation of the ERK pathway (Lim et al., 2014). Additional negative feedback mechanisms also involve ERK-induced transcription of genes encoding Map Kinase Phosphatases (MKPs), which dephosphorylate the activation ERK of loop and attenuate the strength of signalling.

In summary, ERK activation in the cytoplasm might be responsible for indirect regulation of transcription factors. Furthermore, ERK might play a key role in modulating short-term adaptations of the MAPK-ERK signalling pathway using cytoplasmic negative feedback loops. Assuming that GluN2A blockade reduces ERK activation in the cytoplasm post-reactivation, our experiment suggests an important role of the MAPK-ERK signalling pathway in modulating the process of memory restabilisation.

5. Methodological limitations

Limitations

Western-blot is one of the most efficient methods to detect the relative concentration of specific proteins within a sample based on detection with antibodies of interest. Nevertheless, the extent of antibody binding to the specified protein has been shown to be sensitive to experimental errors resulting from complex, multistep manipulations. For example, this method requires that each sample contain the same amount of total protein. While it seems relatively difficult to control the exact protein content of a sample, we have taken this constraint into account by adjusting the dilution factor of our Laemmli and Lysis buffers relative to the protein concentration of each sample.

Second, the comparison of samples based on the protein of interest/loading control ratio is not unproblematic. This process of normalization assumes that loading control proteins are stable under most circumstances. However, it appears that levels of protein can differ between groups depending on the

experimental manipulation (Aldridge et al., 2008). Specifically, β-actin expression has been shown to increase significantly after spinal cord injury (Liu & Xu, 2006). Although the stability of β-actin has not been specifically tested in the context of brain injury, it is important to note that the insertion of the cannula in the lateral amygdala could induce a change in β-actin concentration, making it an unsuitable candidate for normalization. In our experiment, all rats underwent surgery. Accordingly, any increase in β-actin concentration resulting from injury would likely be consistent across our samples. In consequence, comparison between samples would not be significantly impacted by a trauma-induced change in β-actin concentration.

Variability in quantifying proteins of interest with Western-Blot is widely acknowledged. Experimental manipulations, human errors, choices of dilution, incubation time, normalization, types of antibody or concentration of loading control all have an influence on the final quantification. Another important aspect to keep in mind is that labelled enzymes used for chemiluminescence tend to diverge slightly from linearity in terms of signal to noise ratio (LI-COR, 2015). Hence, Western-Blot is considered as a semi-quantitative method.

Addressing Western-Blot limitations

A first step to increase reliability of Western-Blot results is to control for differences in protein loading using either bicinchoninic acid assay (BCA) or Bradford assay and to discard outliers, as performed here. Statistical difference between loading control and within samples should be compared using a standard curve to adjust dilution. Second, samples should be positioned randomly on the gel and gels should be run in duplicate or triplicate to test for differences in loading. This step ensures that optical density values are correlated with protein content for the chosen concentration. These two processes were strictly respected in our experiment.

In lieu of β-actin, Dittmer & Dittmer (2006) have suggested the use of GAPDH as a loading control. Changes in band densities appear visible at lower protein concentration for GAPDH. In the case of low-abundance proteins such as ERK, it could be beneficial to compare the duplicated results obtained with β-actin with a second set of testing with GAPDH to reduce random noise in the data.

At last, both positive and negative controls can improve reliability of the data. A positive result in reaction to a lysate from a tissue sample known to express ERK would indicate that the procedure is valid even if the samples test came back negative. Similarly, a negative control using a null cell line could be used to confirm that protein binding is specific to the protein target (Mahmood & Yang, 2012). These processes are particularly important when Western-Blot is used in the context of clinical virology but also bear implications for scientific research.

Alternatives

Western-Blot is particularly adapted to the detection of target proteins in tissue samples, primarily because of its high specificity and sensitivity. As discussed however, the Western-Blot process is delicate and susceptible to large variability depending on dosage, dilution, concentration and choice of loading control. Other protein-detection techniques present different sets of constraints and advantages.

Fluorescent multiplex Western-Blot uses fluophores for labelling during immunodetection. Proteins of interest can be quantified by measuring the photons emitted by the fluophore in response to excitation by a light source. The use of fluophores reduces variability in quantification by improving the linear dynamic range up to a 10 fold compared to chemiluminescence (McDonald, 2010). Moreover, the use of different photo-stable dyes allows the quantification of multiple proteins in a single experiment, which is particularly useful when looking at signalling interactions or when investigating the role of several receptor types.

Enzyme-linked immune assays (ELISA) use an enzyme to convert a neutral substrate to a coloured product in the presence of antigen-antibody binding. Unlike electrophoresis, which separates the protein of interest based on molecular weight, ELISA use absorbance detection and nucleic acid quantification. Thus, depending on the protein studied, ELISA may be more sensitive to false positive than Western-Blot. Nevertheless, the quantification process appears less versatile than for Western-Blot. The numerical data computed by the ELISA kinetic plate reader is less subjected to variability than the data obtained via Western-Blot image processing software (Jansen et al., 1998).

Of particular interest is the increasing use of flow cytometry in cell signalling profiling. This laser technique measures intact cells or particles based on their light-scattering abilities and fluorescence channels (Krutzik & Nolan, 2003). Light signals are converted into electronic pulses, which are automatically quantified. Flow cytometry presents several advantages over Western-Blot and ELISA. First, it is particularly accurate to quantify kinase activation in contexts where cell numbers are low or sample is limited (Grammer et al., 2004). Second, it is a multivariable method that allows the analysis of several antigens at the cell level. This point bears implications for studies seeking to investigate the role of interactions between multiple signalling pathways or looking at pharmacodynamic modulation. For example, flow cytometry has been used to simultaneously identify the molecular targets of two MEK inhibitors and to demonstrate their effects on pERK activation with great precision (Chow et al., 2001).

Future research

Our findings identify key directions for future research. An analysis of ERK activation in retrieval-condition could be performed after blockade of other subunits of the NMDARs or of other receptors in the basal lateral amygdala. For example, AMPA receptors in both the amygdala and the hippocampus have been shown to play an important role in memory reactivation (Bast et al., 2005; Milton et al., 2013).

Future research in memory retrieval could also take advantage of methodological advances in flow cytometry to study not only the effect of a specific signalling pathway but also the simultaneous activation of several kinases downstream of a receptor subunit, thus providing information on the effects of interacting signalling pathways during reactivation. Other research focusing on memory retrieval have suggested the implication of noradrenaline and calcineurin (Abel & Lattal, 2001; Murchison et al., 2004). The extent to which these various signalling pathways interact with one another during reactivation should provide insights into their specific roles and mechanisms. Specifically, it is hoped that the interaction of kinases involved in regulation of gene transcription during memory retrieval will be identified.

6. Conclusion

To conclude, our experiment could not establish the role of GluN2A NMDARs on ERK activation in the nucleus following reactivation. Instead, we observed a trend whereby ERK was reduced in the cytoplasm of the NVPRet group relative to the VehRet group in the retrieval condition; and conversely, ERK was more concentrated in the NVPNoRet group than in the VehNoRet group in the no-retrieval condition. In addition, NVP-treatment had no noticeable effect on fear-conditioned behaviour in comparison to the control group.

Together, these results suggest that memory reactivation is unlikely to be primarily modulated by GluN2A NMDARs. Nevertheless, the reversed patterns observed in the cytoplasm in the retrieval and no-retrieval conditions indicate that GluN2A NMDARs may play a role in modulating short-term adaptations of the signalling pathway during reactivation. The advantages and limitations of Western-Blot and alternative techniques are discussed in consideration of future research opportunities.

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