Mismatch novelty exploration training impairs $VPAC_1$ receptor mediated modulation of hippocampal synaptic plasticity by endogenous VIP.

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[1]

Highlights:

- Mismatch novelty training (NT) enhances hippocampal CA1 LTP and depotentiation.
- Endogenous VIP $VPAC_1$ receptor modulation of synaptic plasticity is lessened by NT.
- NT does not influence hippocampal levels of VIP or $VPAC_1$ receptors.
- NT and VIP likely impact coincident paths to control LTP and depotentiation.

[1] **Abbreviations:** aCSF, artificial cerebrospinal fluid; EPM, Elevated Plus Maze; FT, fixed training; HT, holeboard training; NT, mismatch novelty training; OF, Open-field; TBS, theta-burst stimulation.

Abstract:

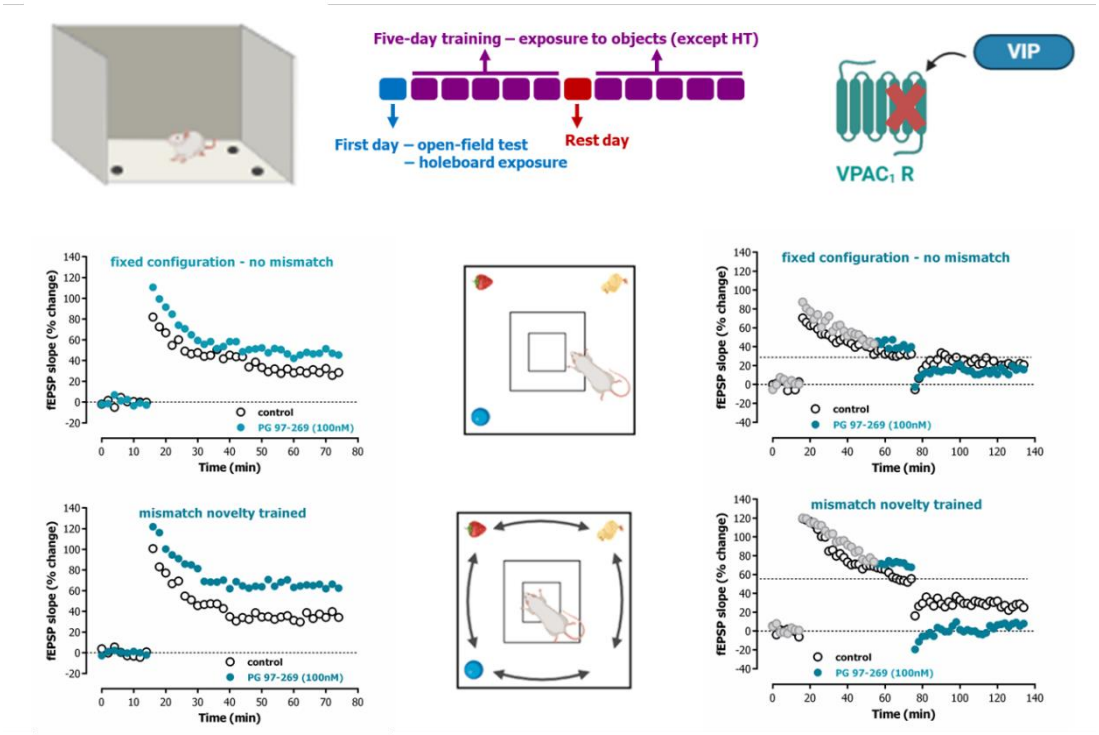
Novelty influences hippocampal-dependent memory through metaplasticity, i.e., experience-dependent adaptations in synaptic plasticity. In this respect, mismatch novelty is known to activate the hippocampal CA1 area in humans and to enhance rat hippocampal-dependent learning and exploration. Mismatch novelty training (NT) by varying spatial configuration of objects in a known environment, enhances rat hippocampal synaptic plasticity. Prefrontal cortex GABAergic projections targeting hippocampal VIP interneurons promote exploration. Since VIP, acting on $VPAC_1$ receptors, restrains both hippocampal LTP and depotentiation by modulating disinhibition we now investigated the impact of NT on $VPAC_1$ receptor modulation of hippocampal synaptic plasticity.

NT enhanced both CA1 hippocampal long-term potentiation (LTP) and depotentiation. Blockade of VIP $VPAC_1$ receptors with PG 97-269 (100nM) enhanced both LTP and depotentiation in naïve animals but was much less effective in enhancing LTP and depotentiation in NT rats. Altered endogenous VIP modulation of LTP was absent in animals exposed to the empty environment (HT). Modulation of depotentiation by endogenous VIP was absent in animals exposed to a fixed configuration of objects (FT) or in HT animals. HT and FT animals, but not NT; showed mildly enhanced synaptic $VPAC_1$ receptor expression.

Altogether this suggests that NT influences hippocampal synaptic plasticity by reshaping brain circuits modulating disinhibition and its control by VIP-expressing hippocampal interneurons. Also, upregulation of VIP $VPAC_1$ receptors maintains VIP control of LTP in FT and HT rats, the absence of this in NT rats leading to enhanced LTP but not influencing depotentiation. This may be relevant to co-adjuvate cognitive recovery therapies in aging or epilepsy, where LTP/LTD imbalance occurs.

Keywords: mismatch novelty, cognitive training, VIP, $VPAC_1$ receptors, LTP, depotentiation, memory, hippocampus, disinhibition, interneurons.

Graphical abstract:



Impairment of endogenous VIP $VPAC_1$ receptor modulation of hippocampal CA1 LTP and depotentiation upon mismatch novelty training.

1. Introduction:

Learning and memory involve experience-triggered adaptations in long-term potentiation (LTP), long-term depression (LTD) or depotentiation of synaptic communication. These are influenced by recent learning experiences or synaptic activity through metaplasticity (Abraham and Bear, 1996; Izquierdo et al., 2001). LTP underlies hippocampal dependent memory acquisition while LTD complements LTP, promoting novelty acquisition and memory reformulation and reconsolidation, required for lasting memory storage during spatial memory formation (Manahan-Vaughan and Braunewell, 1999; Ge et al., 2010; Alberini and Ledoux, 2013; Dong et al., 2013). LTD and LTP encode distinct aspects of novelty acquisition, LTD being enhanced by novel objects or novel object location, and LTP facilitated by new environments, that occlude LTD (Kemp and Manahan-Vaughan, 2004). Depotentiation, in turn, mediates reversal of LTP preceding memory reformulation/erasure and may undergo metaplastic control by prolonged or repeated novelty exposure (Qi et al., 2013).

Mismatch novelty, defined by a conflict between expected and perceived temporal sequence or spatial location of items or events, activates the hippocampal CA1 area in human studies and may be a relevant stimulus for cognitive stimulation (Thakral et al., 2015; Trempler et al., 2017). Animal studies revealed that a mismatch in observed vs expected scenarios triggers either memory reconsolidation or extinction (Pedreira et al., 2004). Mismatched location of known objects enhances inhibitory avoidance learning and memory stability (Dong et al., 2012), favouring hippocampal LTD. A recently developed cognitive stimulus program revealed that mismatch novelty exploration training enhances both hippocampal LTP and depotentiation (Aidil-Carvalho et al., 2017), mildly enhancing hippocampal-dependent learning in young rats (Cunha-Reis, 2020). Such approach could be useful for human memory rescue in aging and diseases where LTP/LTD is imbalanced or near saturation, such as temporal lobe epilepsy (TLE) (Aidil-Carvalho et al., 2017; Cunha-Reis et al., 2021).

Vasoactive intestinal peptide (VIP), an important neuromodulator in the central and peripheral nervous system, has yet important neurotrophic and neuroprotective actions (Ribeiro et al., 2001; Cunha-Reis et al., 2021). In the hippocampus VIP is only present in interneurons of three distinct subtypes, hippocampal basket cells and two interneuron-selective interneuron populations (Acsády et al., 1996). VIP controls GABA release, GABAergic transmission and pyramidal cell activity (Cunha-Reis and Caulino-Rocha, 2020). Activation of two selective receptors, $VPAC_1$ and $VPAC_2$, mediates endogenous VIP actions. $VPAC_1$ receptors mediate the inhibition of hippocampal LTP, LTD and depotentiation by endogenous VIP (Cunha-Reis et al., 2014; Caulino-Rocha et al., 2022) through GABAergic-dependent mechanisms, suggesting the recruitment of distinct VIP-expressing interneurons by different behavioural states and a major influence on cognition. A subpopulation of VIP expressing interneurons is recruited during theta oscillations (Luo et al., 2020) hinting a role in information gating during spatial navigation memory encoding. VIP expressing interneurons are

targeted by *medium raphe* serotonergic and GABAergic projections and septal cholinergic fibres, controlling pacing, engagement, and suppression of hippocampal theta rhythm (Borhegyi et al., 2004; Vandecasteele et al., 2014; Vinogradova et al., 1999) and by prefrontal cortex long-range GABAergic projections (Malik et al., 2022). VIP-mediated hippocampal disinhibition is also crucial for goal-directed spatial learning tasks (Turi et al., 2019) while inhibition of VIP interneurons may enhance feedforward inhibition, facilitating hippocampal object representation during spatial exploration (Malik et al., 2022).

Here, we investigated if the effects of mismatch novelty training on LTP and depotentiation involved a shift in endogenous VIP $VPAC_1$ receptor-mediated modulation of hippocampal LTP and depotentiation.

2. Materials and Methods:

Juvenile (3-4 weeks-old, 120-150g) male Wistar rats were held until use at the local Animal House (22 °C), Faculty of Medicine, University of Lisbon, under a 12:12-h light/dark cycle with food and water *ad libitum*. Before testing, rats were handled for three days. Possible deficits in their motor capacity or altered anxiety levels were evaluated using the open-field (OF) and elevated plus maze (EPM) tests, respectively, to detect deviations that could potentially bias our study. Animals then underwent a two-week training program in mismatch novelty exploration and its impact on hippocampal synaptic plasticity and its modulation by $VPAC_1$ receptors was tested *in vitro* using electrophysiological recordings as described (Aidil-Carvalho et al., 2017). VIP and $VPAC_1$ receptor levels were evaluated by western blot in hippocampal synaptosomes obtained from the contralateral hippocampus. All practices were in accordance with the Guide for Care and Use of Laboratory Animals, the Portuguese and European law on animal welfare (EU Directive 2010/63/EU for animal experiments) and were approved by the Ethical Committee of the Faculty of Medicine. Behavioural testing/training sessions were all performed between 9:00 a.m. and 18:00 p.m. in a sound attenuated room. A preliminary account of some of the results was published as an abstract (Cunha-Reis, 2020).

2.1 Open-field (OF) and Elevated Plus Maze (EPM) pre-evaluation tests.

The OF test consisted of the exploration of a large square chamber (66cmx66cm wide, 60cm-high wall, Fig 1.A), for 5 minutes (Crusio et al., 1989). Three virtual zones (central square 20x20cm, intermediate zone and peripheral 15cm-strip adjacent to the walls) were considered for behaviour analysis. Animals were put directly into the centre of the apparatus and locomotion in the arena was recorded. Animal performance was evaluated by quantifying the escape latency (s), total distance travelled (cm), the number of entries and the time spent in each virtual zone and yet the number of rearings (rat standing on its hind legs, playing or not with his front paws on the wall).

The EPM (Fig. 1.B) was comprised by two open arms (50cmx10cm) and two enclosed arms (50cmx10cmx40cm), extending from a central platform (10cmx10cm) and raised 50cm above floor level. The animal was positioned on the central platform, facing the open arm, and allowed to explore the maze for 5 minutes. The maze was cleaned with a 70% ethanol solution between trials. Each test was video recorded and later analysed with video-tracking software (Anymaze, Stoelting Europe). The number of entries in open/closed arms, time spent in open/closed arms or the central platform, distance (cm) travelled in the maze, and number of rearings were evaluated (Schneider et al., 2011).

2.2 Mismatch novelty training.

Animals undergoing mismatch novelty training (NT, Fig. 1.C-D) were daily exposed for two weeks to three objects (plastic ball, strawberry-shaped rubber and animal-shaped painted wood

figure) always presented in a new location (Aidil-Carvalho et al., 2017) of a holeboard (66x66cm, 60cm-high walled arena containing one hole - 5.5cm diameter, 4.5cm deep- at each corner). As control for novelty training, two animals were exposed to either a fixed object's distribution (FT, Fig. 1.D), or to the holeboard without any object (HT, Fig. 1.D). Naïve animals were left without training. Training was performed in 5-min sessions once a day. Animals explored the empty holeboard on the first day and objects were introduced in three of the four holes for the NT and FT animals on the second day. FT animals found the objects in this same location in the following days, unlike NT animals. Training was performed in two sets of 5-day consecutive training separated by one resting day (Fig. 1.C). Video monitored trials were recorded as a track file using automated video-tracking software (Smart 2.5, PanLab, Barcelona) for later analysis. For each trial, travelled distance, number of entries and time spent in each virtual zone of the apparatus (central, intermediate, and peripheral zones; Fig. 1.D) were evaluated. The number of nose pokes (head dips into the holes) was used to assess object exploration; the number of rearings and distance travelled taken as a measure of general exploratory activity.

2.3 Electrophysiological recordings and synaptic plasticity.

Electrophysiological recordings were performed in hippocampal slices of trained rats 3-5 days after training completion as described (Aidil-Carvalho et al., 2017). Hippocampal slices (400μm) cut perpendicularly to the hippocampus long axis were allowed energetic and functional recovery for 1 h at RT in aCSF (mM: NaCl 124, KCl 3, NaH_2PO_4 1.25, $NaHCO_3$ 26, $MgSO_4$ 1, $CaCl_2$ 2, glucose 10) bubbled with 95% O_2 / 5% CO_2. Each slice was placed in a recording chamber and superfused (3 ml/min) with gassed aCSF at 30.5ºC. Alternate stimulation of two independent sets (S1 and S1) of the Schaffer collateral/commissural fibres was performed every 10 s (rectangular pulses of 0.1 ms) using two bipolar concentric wire electrodes. Field excitatory post-synaptic potentials (fEPSPs) were recorded extracellularly from CA1 *stratum radiatum* using micropipettes (4M NaCl, 2–4 MΩ). Stimulation elicited a fEPSP of 500–850 μV amplitude (about 50% of maximum, similar magnitude for both pathways). The average of six consecutive responses was measured, graphically plotted and recorded for further analysis using the LTP software (Anderson and Collingridge, 2001). fEPSP magnitude was quantified as the slope of the initial phase of the potential.

Independence of the two pathways was confirmed at the end of the experiments by investigating paired-pulse facilitation (PPF) across both pathways. PPF was elicited by two stimuli, with 50 ms pulse interval, delivered sequentially to the two Schaffer collateral pathways (S1 and S2). The P2/P1 ratio between the fEPSP slopes elicited by the second P2 and the first P1 stimuli was computed and compared with the P2/P1 ratio under normal stimulation conditions (10s pulse interval between S1 and S2). Pathway independence was ruled out when PPF of the P2/P1 ratio was larger than 5%.

To elicit LTP, mild theta-burst stimulation (TBS5x4, five trains of 100 Hz, 4 stimuli, separated by 200 ms) was applied. To obtain depotentiation, LTP was first induced by robust theta-burst stimulation (ten trains of 100 Hz, 4 stimuli, separated by 200 ms) and depotentiation induced 1 h later with low-frequency stimulation (LFS, 1 Hz, 15 min). Stimulation protocols used to induce synaptic plasticity were applied after having a stable baseline for at least 20 min. Stimulus intensity was not changed therein. LTP or depotentiation were quantified as the % change in fEPSP slope 50 to 60 min after the induction protocol, in relation the fEPSP slope measured during the 12 min that preceded it. Changes in the induction phase of LTP or depotentiation were quantified in the 4 to 6 min following the induction protocol. Control and test conditions were tested in independent pathways in the same slice. S1 denotes the pathway (left or right) to which TBS5x4 (or TBS10x4 followed by LFS) was first applied. The $VPAC_1$ receptor antagonist, PG 97-269, was added to the perfusion solution 20 min before TBS5x4 or LFS delivery to the test pathway (S2) and kept until the end of the experiment. Each *n* represents a single LTP experiment (S1 *vs* S2 conditions) performed in one slice from an independent animal.

2.4 Western-blot analysis of VIP and $VPAC_1$ receptor levels.

Hippocampal synaptosomes were isolated from the hippocampal tissue of trained animals as previously described (Caulino-Rocha et al., 2022). For western blot studies samples were incubated at 95ºC for 5 min with Laemmli buffer (125mM Tris-BASE, 4% SDS, 50% glycerol, 0,02% Bromophenol Blue, 5% 1,4-dithiothreitol), run on standard 10% SDS-PAGE and transferred to PVDF membranes (0.45 µm pore, Immobilon) (Rodrigues et al., 2021). These were blocked with either 3% BSA or 5% milk and incubated overnight at 4ºC with rabbit polyclonal anti $VPAC_1$ receptor (1:600, Alomone Labs #AVR-001; RRID: AB_2341081), rabbit polyclonal anti VIP (1:300, Proteintech, # 16233-1-AP, RRID: AB_2878233), mouse monoclonal anti-β-actin (1:5000, Proteintech #60008-1-Ig, RRID: AB_2289225) and rabbit anti-α-tubulin (1:5000, Proteintech #11224-1-AP; RRID: AB_2210206) primary antibodies. After washing, membranes were incubated for 1h with anti-rabbit or anti-mouse IgG secondary antibody conjugated with horseradish peroxidase (HRP) (Proteintech) at RT. HRP activity was detected by enhanced chemiluminescence using the ImageQuant™ LAS 500 detector (GE Healthcare). Band intensity was evaluated with the Image J software using β-actin or α--tubulin band density as loading control. $VPAC_1$ and VIP band intensities were normalized to the β-actin or α--tubulin loading controls, respectively.

2.5 Materials:

PG 97-269, (Phoenix peptides, Europe) was made up in 0.1mM stock solution in CH_3COOH 1% (v v-1). The maximal concentration of CH_3COOH delivered to the slices, 0.001% (v/v), induced no

change on fEPSP slope (n=26). Stock solutions were kept frozen at -20ºC in aliquots until use. Aliquots were thawed and diluted in aCSF for use in each experiment.

2.6 Statistics

Values are presented as the mean ± S.E.M. n denotes the number of animals in all Figures. Throughout training significance of the differences in behavioural parameters between the groups (Fig. 1.D-E) was calculated by Two-way analysis of variance (ANOVA) with Sidaks's post hoc test unless otherwise stated. Significance of potentiation or depotentiation against the null hypothesis was evaluated using Student's t-test. Significance of differences caused by different modes of training on LTP and depotentiation were calculated by One-way ANOVA as for the EPM and OF tests using GraphPad Prism 6.01. $P<0.05$ or less denotes significant differences.

3. Results:

3.1 Open-field (OF) and Elevated Plus Maze (EPM) tests.

Analysis of behaviour in the OF, relied on the definition of three virtual zones (peripheral, intermediate, and central zones). Animals spent much less time in the centre (3.7±0.9s, n=26) and intermediate (6.0±1.2s, n=26) zones of the apparatus when compared to the periphery zone (286.7±2.0s, n=26), a behaviour known as thigmotaxis ($F_{(2,75)}$ =12838, P<0.0001, Fig. 1.A). Accordingly, the distance travelled in the centre and intermediate zones (72.6±13.2cm and 123.1±23.5cm, n=26, respectively) of the apparatus was much smaller ($F_{(2,75)}$ =144.9, P<0.0001) when compared to the periphery zone (3174.0±254.2cm, n=26). Animals had very few entries (1-4) in any of the different zones. Global locomotion (accessed by total distance travelled; 3460.2±219.0cm, n=26) was high as usually observed in our lab for juvenile rats. The total number of rearings (9.2±1.6, n=26) reflects mostly rearings performed in the periphery zone.

In the EPM (Fig. 1.B) the % time animals spent in the open arms (19.2±2.3% of a 5-min trial, n=26) was much lower ($F_{(2,75)}$ =124.4, P<0.0001) than the %time spent in the closed arms (63.3±3.0%, n=26). In the remaining time (17.6±1.3%, n=26) the animals remained in the centre of apparatus (the crossing of closed and open arms). Accordingly, all animals entered the open arms less (5.3±0.5, n=26) than the closed arms (8.6±0.4, n=26) from this central position. Rearings (7.3±0.8, n=26) were almost entirely performed within the closed arms.

3.2 Animal training in novel location of known objects.

The two-week NT program (Aidil-Carvalho et al., 2017) consisted 5-min per day training in the novel location of known objects delivered in two series of 5-day training. As a control, animals were exposed either to the holeboard in the absence of objects (HT) or to the known objects every day in a fixed location (FT) of the holeboard. Behaviour was observed throughout the training sessions.

Upon initial exposure to the holeboard (no objects for all animal groups), the general exploratory behaviour was reduced when compared to the one observed in the OF maze. The number of rearings in holeboard exploration (5.1±0.6, n=21), was less than the one observed in the open-field test (9.2±1.6, n=26) and the total distance travelled on the holeboard (2500.8±168.5cm, n=21) was slightly higher than the total distance travelled in the open-field (2252.9±96.0cm, n=26). The animals spent more time in the periphery zone of the holeboard (279.9±6.2s, n=21), where the holes were located, than in the periphery of the OF (233.1±8.0s, n=26). Thus, animals although likely now less fearful, did not spend more time in the intermediate or centre zones, remaining in the periphery exploring the holes, as evidenced by the number of nose-pokes (12.6±1.3, n=21). The next day, when objects were placed in the holes for NT and FT animals, the number of nose-pokes increased to17.0±1.2 (n=7) and 16.3±4.5 (n=7), respectively ($F_{(3,48)}$ = 5.801; P<0.005). This was not observed for the HT group

(11.3±1.7, n=7). No significant differences were encountered in the distance travelled in the holeboard periphery zone as compared with the first exposure to the empty holeboard between for any of the trained groups. The number of rearings decreased mildly for all animal groups, likely due to a diversion of their focus to exploration of the holes instead of the whole holeboard.

A marked enhancement in the total distance and in the distance travelled in the periphery zone of the apparatus was observed throughout the training up to day 10 for all animals (Figure 1.E, $F_{(4,78)}$ = 19,45, P<0.05, two-way ANOVA). No significant differences (P>0.05, $F_{(2,78)}$ = 0.8385, two-way ANOVA) between animal groups were observed throughout training. No interaction between the two variables (training mode and training day) was detected (P>0.05, $F_{(8,78)}$ = 0.3788, two-way ANOVA). The time spent in the periphery of the holeboard decreased significantly throughout training for the HT animal group ($F_{(4,96)}$ = 5.168, P<0.0001, two-way ANOVA) but not for the NT and FT group.

The number of nose-pokes increased significantly throughout training for NT rats ($F_{(4,76)}$ = 2.265, two-way ANOVA, P<0.05) but not for HT or FT animals (Figure 1.F). Nose-pokes increased from 16.0±1.1 (n=7) on the first day to 21.4±1.7 (n=7) on the tenth day of training for the NT group. The number of nose pokes for the FT group (14.8±0.3 on day 1 vs. 17.3±1.3 on day 10, n=7) and for HT group (11.7±2.0 on day 1 vs. 12.3±1.8 on day 10, n=6) were similar. The number of rearings increased significantly from the first to the last day of training for animals undergoing HT and significant differences were observed between animal groups ($F_{(2,76)}$ =8.398; P<0.05, two-way ANOVA).

3.3 Impact of mismatch novelty training on hippocampal synaptic plasticity.

Basal stimulation conditions elicited a fEPSP that was 40-60% of the maximal response in each slice and had an average slope of 0.589±0.032mV/ms (n=40). For synaptic plasticity experiments, when inducing LTP, mild theta-burst stimulation (TBS5x4) was applied to one of the pathways (S1 or S2) that was therein considered the control pathway. In Naïve animals an initial post-tetanic potentiation (PTP, 53.5±2.8% increase in fEPSP slope, n=7, Fig. 2.A) was observed that decayed over time. An LTP was still observed 50-60min after stimulation, as a 24.6±2.3% increase in fEPSP slope (n=7, P<0.05, t-test, Fig. 2.A). This LTP was fully dependent on NMDA receptor activity, as previously described by others, since it was abolished (P>0.05, t-test, n=4) when the NMDA receptor antagonist AP-5 (100μM) was present in the medium from 20 min before TBS5x4 stimulation. As previously described, when the $VPAC_1$ receptor antagonist was added to the slices 20 min before TBS5x4 delivery to the test pathway, PTP was higher than in the control pathway (81.5±9.5% increase in fEPSP slope) and the observed LTP 50-60 min post stimulation was also enhanced as it induced a 45.5±6.5% increase (n=7, Fig. 2.A) in fEPSP slope, as previously described (Caulino-Rocha et al., 2022). PG 97-269 (100nM), when added to the slices, did not change the fEPSP slope as estimated in the non-stimulated pathway.

As previously described, when TBS5x4 was applied to hippocampal slices obtained from NT rats, an enhancement ($F_{(3,22)}$ =10.72, P<0.005) of LTP as observed 50-60min post stimulation, with TBS5x4 now causing a 35.8±1.5% increase in fEPSP slope (n=7, Fig. 2.D). Yet, such an enhancement was absent in slices from HT or FT animals, since we encountered a similar LTP magnitude to the one observed in Naïve rats (24.2±1.8% n=6, Fig. 2.B and of 27.8±2.1% n=6, Fig. 2.C enhancement in fEPSP slope for HT and FT, respectively). This difference in the response to TBS5x4 was evident early after LTP induction since 4-6 min post stimulation with TBS5x4 the PTP in NT animals was significantly higher (68.2±4.5% increase in fEPSP slope) than the one observed in Naïve and HT animals (51.7±3.3% and 44.6±4.5% increase in fEPSP slope, respectively).

When PG 97-269 (100nM) was added to the slices 20 min before stimulation with TBS5x4 in the test pathway, the enhancement on LTP in HT and FT was similar to the one observed in Naïve animals (43.3±2.6% n=6, Fig. 2.B and of 46.5±6.1% n=6, Fig. 2.C, enhancement in fEPSP slope for HT and FT, respectively) and nearly doubled the one obtained in the absence of the $VPAC_1$ antagonist. Conversely, endogenous VIP modulation of hippocampal LTP was impaired by mismatch novelty training ($F_{(7,44)}$ =7.841, P<0.0001) since LTP elicited by TBS5x4 in the presence of PG 97-269 (100nM) was only about 50% larger (54.6±4.8% increase in fEPSP slope, n=7, Fig.2.D) than the one observed in the absence of the $VPAC_1$ antagonist for NT animals. This effect was even more pronounced in the early moments post-induction, since no significant effect was observed in NT animals between the PTP obtained in the control pathway (absence of drugs, % increase in fEPSP slope 68.2±4.5, n=7) and the one obtained in the test pathway (presence of PG 97-269 100nM, % increase in fEPSP slope 89.2±6.5, n=7) unlike what was observed in Naïve, HT and FT animals ($F_{(7,44)}$ = 7.473, P<0.0001).

Low-frequency stimulation (LFS) delivered to hippocampal slices of adult rats *in vitro* does not produce an LTD of CA1 synaptic transmission in the absence of added drugs (Wagner and Alger, 1995; Cunha-Reis et al., 2014). As such, to induce long-lasting depression of synaptic transmission LFS was applied 1h after delivering a TBS10x4 stimulus. In Naïve animals, a depotentiation of synaptic transmission was immediately observed after LFS stimulation (% decrease in fEPSP slope 20.4±1.8%), that was partially maintained 50-60min after (12.2±1.4% decrease in fEPSP slope, n=5, P<0.05, Fig. 3.A). As previously described (Cunha-Reis et al., 2014), when LFS was delivered in the presence of the VIP $VPAC_1$ receptor antagonist PG 97-269 (100nM) the depotentiation was twice as large (26.2±1.0% decrease in fEPSP slope, n=5, P<0.05, Fig. 3.A).

When the same stimulation pattern was delivered to hippocampal slices obtained from NT animals (Fig. 3.D) a larger depotentiation was observed ($F_{(3;24)}$ =8.90; P<0.0005), since LFS delivered 1h after TBS10x4 now caused a decrease of 23.1±1.7% (n=7) in fEPSP slope. This enhancement was not observed for HT and FT animals, since depotentiation magnitude was comparable to the one obtained in Naïve animals 50-60min after LFS (% decrease in fEPSP slope of 15.3±1.5% n=7, Fig. 3.B and of

15.8±1.5% n=7, Fig. 3.C, respectively). This difference in the response to LFS was not evident early after LTD induction since 5 min post stimulation the depression in fEPSP slope in NT animals was not significantly higher than the one observed in Naïve, HT and FT animals ($F_{(3,24)}$ =1.61, P>0.05, Fig. 3.E).

When PG 97-269 (100nM) was added to the slices 20 min before LFS of the test pathway, the enhancement on depotentiation in HT and FT was not altered, being only slightly higher than the one observed in Naïve animals in the absence of PG 97-269 (18.8±2.2% n=7, Fig. 3.B and of 20.6±2.9% n=7, Fig. 3.C, decrease in fEPSP slope for HT and FT, respectively). Conversely, endogenous VIP modulation of hippocampal CA1 depotentiation was impaired by mismatch novelty training ($F_{(7,44)}$ =7.661, P<0.0001) since depotentiation elicited by LFS in the presence of PG 97-269 (100nM) was only about 40% larger (32.6±3.1% increase in fEPSP slope, n=7, Fig.3.D) than the one observed in the absence of the $VPAC_1$ antagonist for NT animals.

3.4 Impact of mismatch novelty training on expression of hippocampal VIP and synaptic VIP $VPAC_1$ receptors.

To determine in differences in $VPAC_1$ modulation of hippocampal synaptic plasticity in the hippocampus could be due to training-induced adaptations in $VPAC_1$ receptor levels we performed western blot experiments in hippocampal synaptosomes obtained from the hippocampus of NT trained rats and respective naïve and functional training controls (HT and FT animals). $VPAC_1$ receptor levels nearly doubled in HT rats since we observed a 72.9±13.6% increase in $VPAC_1$ receptor immunoreactivity (n=6, Fig 4). We also observed a very mild increase (27.9±7.6%, n=6, Fig 4) in $VPAC_1$ receptor levels in FT animals. $VPAC_1$ receptor levels were not significantly altered in NT rats (n=6, $F_{(3,20)}$ =0.3608, P>0.05, Fig. 4.B), thus suggesting that modified control of synaptic plasticity in NT animals is not due to altered $VPAC_1$ receptor levels. To determine if altered endogenous VIP levels in hippocampal nerve terminals could account for this effect, we also performed WB experiments to quantify VIP synaptic levels. VIP was mildly enhanced in synaptosomes obtained from HT animals (23.1±6.7%, n=5, Fig 4) but not in FT or NT animals, for which a mild tendency to decreased VIP levels was observed, although not reaching statistical significance, when compared to Naïve animals (n=5, $F_{(3,16)}$ =2.161 and 1.800, respectively, P>0.05, Fig. 4.D).

4. Discussion:

The main findings of the present work are that training of young adult Wistar rats in exploration of the novel location of known objects in a holeboard for two weeks induces an impairment in the ability of endogenous VIP to inhibit LTP and depotentiation in hippocampal CA1 area, an effect that is dependent on activation of $VPAC_1$ receptors. This shift does not appear to involve substantially altered synaptic VIP and $VPAC_1$ receptor levels in novelty trained rats. Altogether, these observations suggest that altered control of hippocampal synaptic plasticity by VIP $VPAC_1$ receptors following mismatch novelty training is mainly related to alterations either in the targets of VIP immunoreactive interneurons, or in global network activity. Conversely, HT caused a mild enhancement of VIP in the hippocampus and a considerable increase in $VPAC_1$ receptor levels. Although this could suggest a potential role in enhanced inhibition of synaptic plasticity events in the hippocampus, this was not observed in hippocampal CA1 synaptic plasticity. Nevertheless, changes in synaptic plasticity in other hippocampal areas may also occur that can be related to these altered $VPAC_1$ levels. These findings provide a first insight on a possible mechanism for the selective enhancement of hippocampal synaptic plasticity by mismatch novelty training, the regulation of hippocampal circuits also targeted by interneurons expressing VIP. This could be useful to develop combination therapies (cognitive together with pharmacological) for cognitive decline in aging or epilepsy.

Novelty can relate to different aspects in exploration of the environment that have been described to influence cognition in different ways (Izquierdo et al., 2001; Dong et al., 2012; Quent et al., 2021). Mismatch novelty detection is vital for adaptation to novel situations in an otherwise familiar environment or to detect inconsistencies in previously acquired memories. As such, it is not surprising that it primes the brain for cognitive flexibility and learning-associated plasticity (Moncada and Viola, 2007; Park et al., 2021; Quent et al., 2021). This makes it the ideal cognitive training paradigm to elicit changes in synaptic plasticity and cognition, but its underlying mechanisms need to be further elucidated.

In this work, when initiating the training, the rats were first exposed to the empty holeboard. This triggers a behavioural response to the novel environment that reflects a combination of curiosity and fear (Kanari et al., 2005) given that not only general exploratory activity, as assessed by the number of rearings, was decreased when compared to exposure to the OF but also that locomotion in the periphery was slightly enhanced. This likely reflects also an interest in the holes, as evidenced by the number of nose-pokes (Brown and Nemes, 2008; Aidil-Carvalho et al., 2017). The presence of objects in the holes on the second day of training for NT and FT animals introduced an added novelty and, as expected, induced an enhancement in the number of nose-pokes for both the NT and FT groups but not the HT group, re-exposed to the holeboard, suggesting that the presence of the objects is driving this increase in head-dipping (Aidil-Carvalho et al., 2017). The distance travelled in the periphery (but

not the intermediate and central zones) of the holeboard increased along training for NT animals (Fig 1), indicating an enhanced interest in object/hole exploration over holeboard space exploration. Conversely, HT and FT animals demonstrated an increase in general exploration, estimated from the time spent in the intermediate and central zones and number of rearings, confirming that novel object configurations drive enhanced interest in object exploration (Goh and Manahan-Vaughan, 2013b; Aidil-Carvalho et al., 2017).

Mismatch novelty training induced an enhanced LTP response to TBS5x4 stimulation and enhanced depotentiation response to LFS in NT animals but not HT or FT animals as shown (Aidil-Carvalho et al., 2017). Preliminary work from our Lab suggests it can also improve spatial cognition, especially in experimental conditions associated with altered LTP/LTD ratios like aging and epilepsy (Amaro-Leal et al., 2016; Cunha-Reis, 2020). This suggests that this mismatch novelty training program may induce persistent changes at either synaptic domains or hippocampal circuits, favouring those altered responses to TBS or LFS. Possible mechanisms may involve altered physiology of hippocampal VIP expressing interneurons, that are targeted by many extrahippocampal projections regulating cognition during exploration and novelty/mismatch novelty responses. In fact, a subpopulation of VIP expressing interneurons recruited during theta oscillations (Luo et al., 2020) may play a role in information gating during spatial navigation and memory encoding. VIP interneurons are targets of *medium raphe* serotonergic and GABAergic projections and septal cholinergic fibres, that regulate the pacing, initiation, and suppressing of hippocampal theta rhythm (Borhegyi et al., 2004; Vandecasteele et al., 2014; Vinogradova et al., 1999). Furthermore, they are also targeted by prefrontal cortex long-range GABAergic projections (Malik et al., 2022), implicated in triggering hippocampal theta oscillations in response to mismatch computations (Garrido et al., 2015). As such, VIP expressing interneurons are in position to regulate many aspects of hippocampal dependent cognition related to mismatch novelty detection and processing.

VIP-mediated hippocampal disinhibition, being crucial for goal-directed spatial learning tasks (Turi et al., 2019) while suppressed to allow the hippocampal representation of objects during spatial exploration (Malik et al., 2022) could be differentially regulated by distinct types of novelty, known to trigger different levels of arousal and to selectively enhance activation of the hippocampus by serotonergic, noradrenergic, cholinergic and dopaminergic inputs (Walling et al., 2011; Schiffer et al., 2012; Schomaker and Meeter, 2015; Hagena and Manahan-Vaughan, 2017; Moreno-Castilla et al., 2017). These pathways are also involved in learning from prediction errors in humans (Schiffer et al., 2012), and differentially enhance/inhibit hippocampal synaptic plasticity in response to multiple behavioural stimuli (Hagena and Manahan-Vaughan, 2017). VIP immunoreactive interneurons control both feedforward and feedback inhibition being in a position to effectively modulate bidirectional hippocampal synaptic plasticity (Cunha-Reis and Caulino-Rocha, 2020). This, together with our recent

findings that VIP $VPAC_1$ receptor blockade could simultaneously enhance both hippocampal LTP and depotentiation (Cunha-Reis et al., 2014; Caulino-Rocha et al., 2022), led us to hypothesize that mechanisms controlled by VIP expressing interneurons could mediate, at least in part, the enhancing effects of mismatch novelty training on hippocampal LTP and depotentiation (Aidil-Carvalho et al., 2017). The observations in this work that mismatch novelty training does not change VIP or $VPAC_1$ receptor levels in hippocampal synaptosomes, and that $VPAC_1$ receptor mediated modulation of both LTP and depotentiation is impaired by mismatch novelty training appear to support this view. In particular, repeated inhibition or activation of distinct VIP-immunoreactive interneurons by mismatch novelty could favor an adaptation of synaptic communication and a decrease in the threshold for LTP and depotentiation induction. A particular VIP-expressing interneuron subtype described to date only in mice, VIP long-range projection interneurons, was reported to be active only when the hippocampal theta rhythm is supressed (Francavilla et al., 2018), as happening during attention shifts caused by novelty detection (Kitchigina et al., 1999). If present in the rat may be responsible for VIP modulation of depotentiation. Altogether, these observations are consistent with our previous findings that basal synaptic transmission is decreased in NT animals (Aidil-Carvalho et al., 2017), that both LTP and depotentiation are enhanced in NT animals, and previous findings that mismatch novelty favors LTD and depotentiation over LTP induction *in vivo* (Kemp and Manahan-Vaughan, 2004; Sachser et al., 2017). This may be of particular relevance since depotentiation *in vivo* may be crucial for memory reformulation following mismatch detection (Goh and Manahan-Vaughan, 2013a).

The impaired effects of the $VPAC_1$ antagonist on LTP/depotentiation in NT animals could be due to reaching a ceiling effect on LTP/depotentiation expression by their enhancement in response to training alone. Given that we used a mild TBS stimulation to induce a moderate LTP, and that the depression of synaptic transmission induced by this protocol is mild, it is not likely that the reduced effects of the $VPAC_1$ antagonist on LTP were due to near saturation of LTP or depotentiation mechanisms. Furthermore, as discussed above, the possibility that mismatch novelty training might have induced an adaptation in the activity of VIP immunoreactive interneurons or their interneuron targets is also not to be excluded and should be further investigated.

The changes observed in VIP and $VPAC_1$ receptor levels in HT and FT rats do not fully align with the observed effects on $VPAC_1$ modulation of LTP and depotentiation. Being obtained from the full hippocampus and not only from the CA1 area, where changes in synaptic plasticity were studied, the sinaptossomal preparation used to quantify VIP and $VPAC_1$ receptor levels may have masked the effects of mismatch novelty training on VIP or $VPAC_1$ receptors in the CA1 area and the elucidation of this mechanism may require a different experimental approach. Regardless, the CA1 area of the hippocampus is known to be specifically activated by both spatial and temporal mismatch novelty in

humans (Duncan et al., 2012; Chen et al., 2015; Thakral et al., 2015), and has for long been implicated in mismatch novelty detection and processing (Vinogradova, 2001).

In conclusion, endogenous VIP, present on VIP immunoreactive interneurons, and acting on $VPAC_1$ receptors, appears to be an important component of hippocampal-dependent mismatch novelty processing. The actions of VIPergic system can be altered by mismatch novelty training in a way that is consistent with the use of concurrent mechanisms to modulate CA1 hippocampal LTP and depotentiation. Conversely, repetitive training, especially in the absence of objects, induced an enhancement of $VPAC_1$ receptor expression that may not relate to functional changes in the CA1 area.

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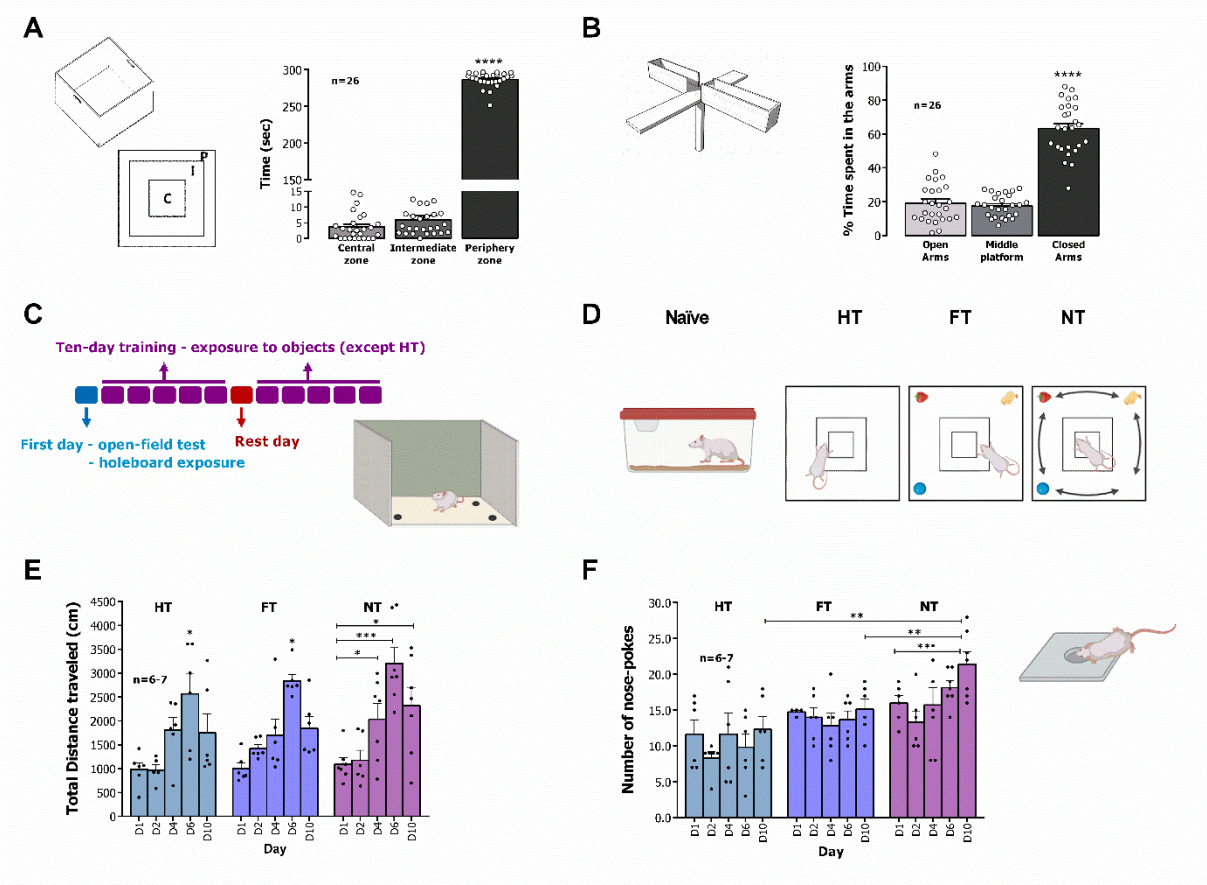

Figure 1 – Mismatch novelty training paradigm and behavioural analysis. A. Evaluation of motor and exploratory capacity in the open-field (OF) test. ***p<0.005 (One-way ANOVA) as compared to central zone. **B.** Evaluation individual anxiety levels in the elevated plus maze (EPM) test. ****p<0.001 (One-way ANOVA) as compared to %time in open arms. **C.** Time-course of the two-week mismatch novelty training and illustration of the holeboard arena used to conduct it. **D.** Mismatch novelty paradigm (NT) in object exploration in a known environment and respective functional controls: exploration of a fixed configuration of objects (FT) or the empty holeboard (HT, holes not shown). Naïve animals remained every day in their cages. Total distance travelled (**E.**) and number of nose pokes (**F.**) during the training by animals undergoing different training modalities (HT, FT and NT). Values are mean ± S.E.M. *p < 0.05, **p < 0.01 and ***p<0.005 (Two-way ANOVA) as compared to D1.

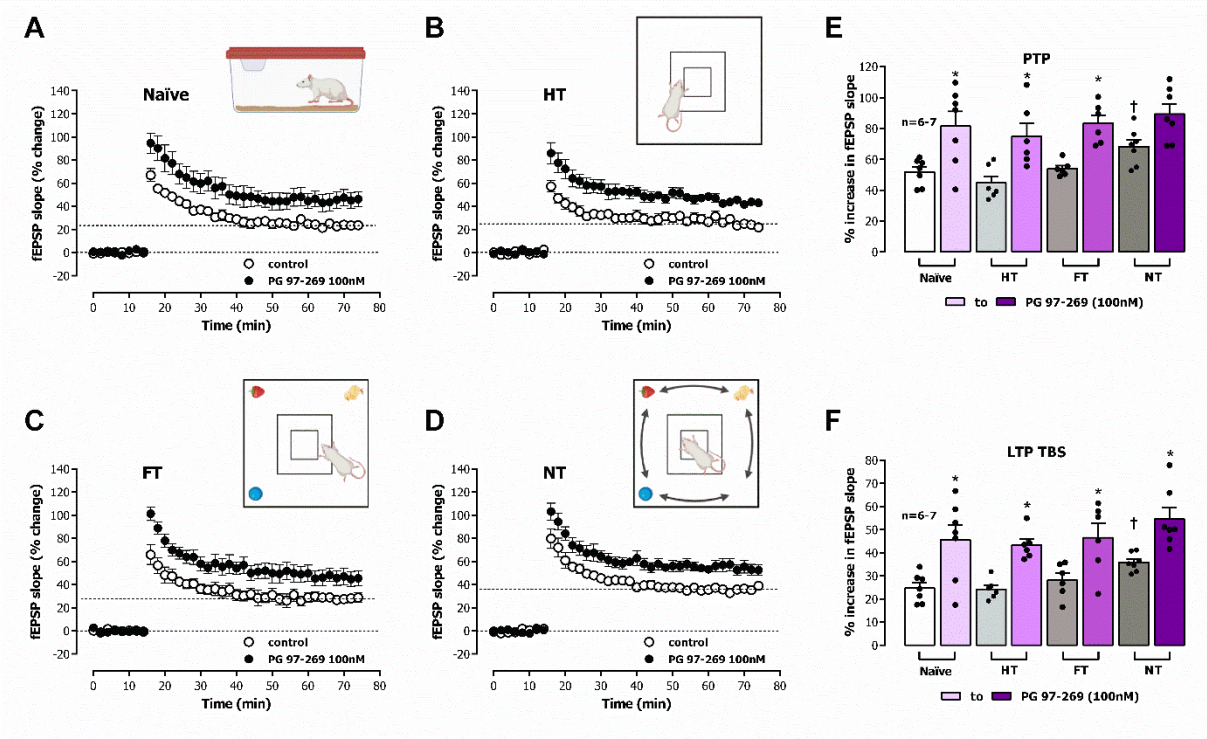

Figure 2 – Impact of mismatch novelty training on VPAC$_1$ modulation of TBS-induced CA1 LTP by endogenous VIP. Averaged time-course of changes in fEPSP slope caused by theta-burst stimulation (5x 100 Hz bursts, 200ms interval, TBS5x4) in hippocampal slices from Naïve (**A.**), holeboard trained (HT, **B.**), fixed object trained (FT, **C.**) and novelty trained (NT, **D.**) animals in the absence (-o-) and in the presence (-•-) of the VPAC$_1$ receptor antagonist PG 97-269 (100nM). Initial potentiation (PTP, **E.**) and magnitude of the LTP (**F.**) estimated from the averaged increase in fEPSP slope observed 4-6 and 50-60 min after TBS5x4 in control conditions for Naïve animals (open bar) and left to right HT (light grey), FT (median grey) and NT (dark grey) animals, and respective PTP (**E.**) and LTP magnitude (**F.**) obtained in the presence of PG 97-269 (100nM) in Naïve animals (light pink) and, left to right, HT (lavender), FT (lilac) and NT (violet) animals. Values are mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ and ***$p<0.005$ (one-way ANOVA) as compared to PTP (**E.**) or LTP (**F.**) obtained in the absence of PG 97-269 for each training modality (next column in the left).

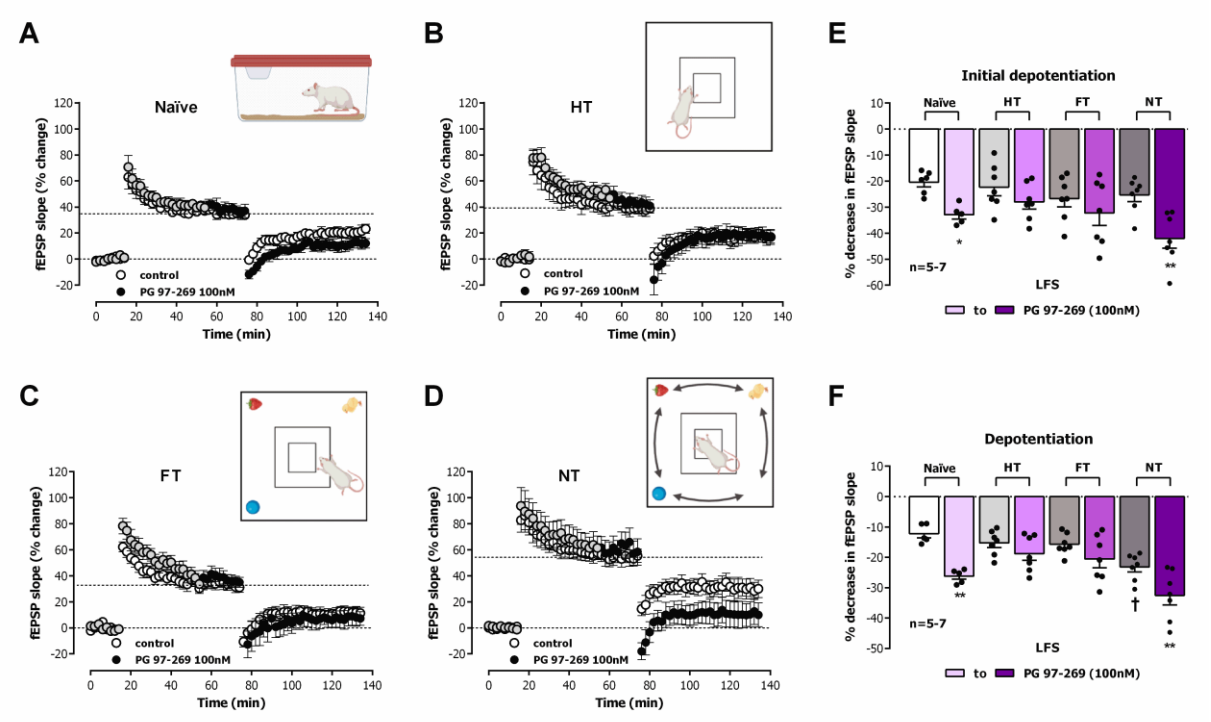

Figure 3 – Impact of mismatch novelty training on $VPAC_1$ modulation of CA1 depotentiation by endogenous VIP. Averaged time-course of changes in fEPSP slope caused TBS10x4 followed 1 hour later by LFS (1Hz, 15min) in hippocampal slices from Naïve (**A.**), holeboard trained (HT, **B.**), fixed object trained (FT, **C.**) and novelty trained (NT, **D.**) animals in the absence (-o-) and in the presence (-•-) of the $VPAC_1$ receptor antagonist PG 97-269 (100nM) from 20 min before LFS. Initial depression (**E.**) and depotentiation magnitude (**F.**) of the fEPSP slope observed 4-6 and 50-60 min after LFS for Naïve animals (open bar) and left to right HT (light grey), FT (median grey) and NT (dark grey) animals, and respective initial depression (**E.**) and depotentiation magnitude (**F.**) obtained in the presence of PG 97-269 (100nM) in Naïve animals (light pink) and left to right HT (lavender), FT (lilac) and NT (violet) animals. Values are mean ± S.E.M. *$p < 0.05$, **$p < 0.01$ and ****$p<0.001$ (one-way ANOVA) as compared to the early potentiation (**E.**) or LTP (**F.**) obtained in the absence of PG 97-269 for each training modality (next column in the left).

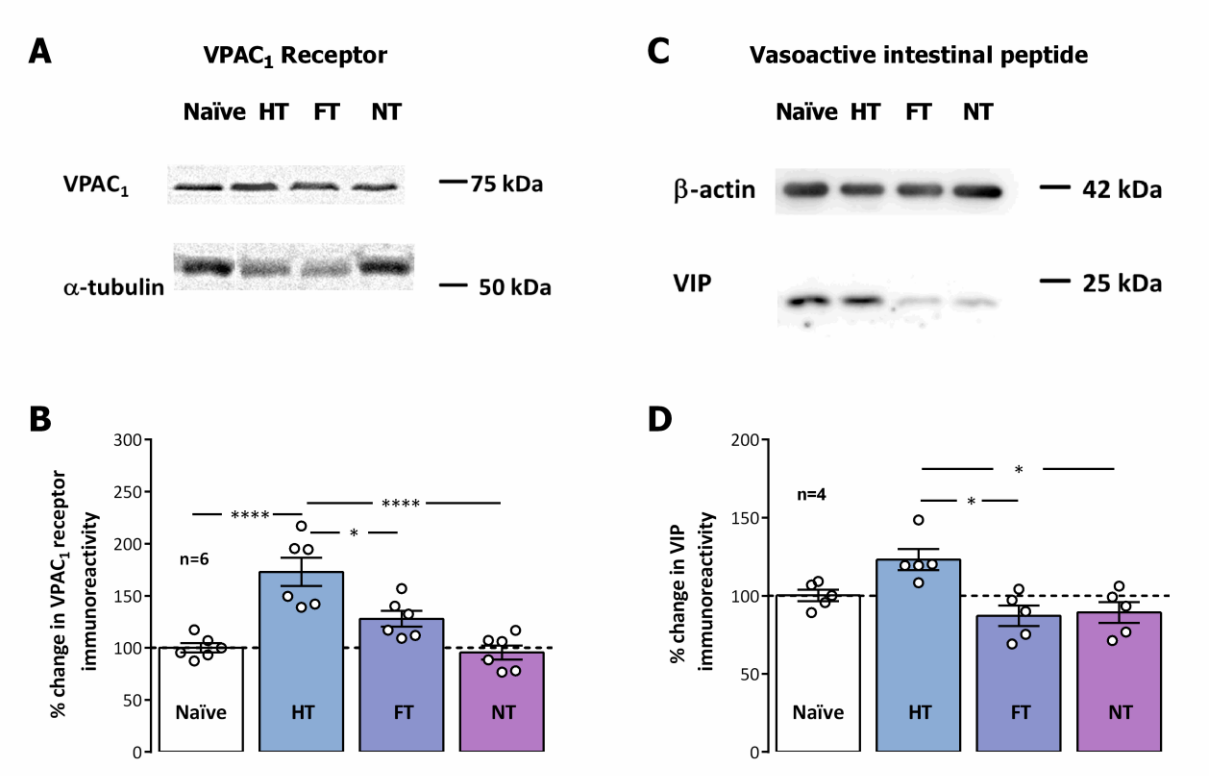

Figure 4 – Impact of mismatch novelty training on VIP and VIP $VPAC_1$ receptor levels in hippocampal synaptosomes. Individual experiment showing western-blot immunodetection of VIP $VPAC_1$ receptors (**A.**) and respective α-tubulin loading control or VIP and respective β-actin loading control (**C.**). **B.** Increased levels of $VPAC_1$ receptor levels (**B.**) and mildly increased VIP levels (**D.**) observed in HT rats as shown by Western blot immunodetection in hippocampal synaptosomes. Values are mean ± S.E.M of 4-5 independent experiments performed in duplicate. 100% - averaged target immunoreactivity obtained for Naïve rats. *$p < 0.05$ and **$p < 0.01$ (One-way ANOVA followed by Sidak's multiple comparison test).