NMDA Phosphorylation in Olfactory Preference Learning

A critical step in neuronal plasticity?

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Abstract

Neonate rats (PND 7) exposed to peppermint odour paired with tactile stimulation (i.e., stroking) exhibit an approach preference to the odour when tested 24 hr later. This conditioning is also found when an optimal dose of β-adrenergic agonist isoproterenol, but not suboptimal or excessive doses, is substituted for stroking as the unconditioned stimulus. Serotonin (5-HT) appears to modulate this effect, as rats depleted of bulbar 5-HT require a higher optimal dose of isoproterenol to learn an approach preference for the odour. β_1 and 5-HT_{2A} receptors colocalize in these cells and appear to coregulate the production of cAMP, which is strongly implicated in the formation of long-term potentiation (LTP) in many learning models. Evidence from other learning paradigms, particularly those involving the hippocampal CA1 region, suggest that β_1 activity may act to regulate the behaviour of NMDA- and AMPA-type glutamate receptor functioning, and this is a putative point of convergence for the conditioned and unconditioned stimuli in the present model. Electrophysiology in neonatal rat olfactory bulb indeed indicates an increased amplitude of both the NMDA and AMPA components of olfactory nerve evoked field potentials (ON-EFPs) during learning acquisition, but only in response to learning-effective doses of isoproterenol. The present study utilizes Western blotting techniques to investigate the possibility that the Ser897 site of the NR1 subunit of NMDA is being phosphorylated during this interval. Ser897 has been shown to be selectively phosphorylated by protein kinase A (PKA), and this enzyme is directly activated by cAMP. Results indicate that Ser897 is indeed phosphorylated at 10 min following training, but only in rats receiving isoproterenol paired with odour. These findings are

despite the failure of the present study to replicate the odour approach conditioning found by numerous others. Implications of these findings to the emerging model of olfactory learning are discussed.

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Odour Approach Preference Conditioning

In a simple classical conditioning paradigm, neonatal rats exposed to peppermint odour as the conditioned stimulus (CS) paired with tactile stimulation (i.e., stroking) as the unconditioned stimulus (UCS) demonstrate an approach preference to the odour when tested 24 hr later. This preference is not observed in rats exposed to either the CS or UCS alone, or both conditions applied separately (Sullivan *et al.*, 1994). Olfactory memory is important in neonatal rats, as it seems to facilitate crucial interactions with the mother, such as feeding, that are integral to survival (Sullivan *et al.*, 1991; Wilson *et al.*, 1985). In these rats, stroking appears to activate the nucleus locus coeruleus (LC), a group of noradrenergic (NE) neurons located in the brainstem (Nakamura *et al.*, 1987). Lesions of LC in rat pups by 6-OHDA have resulted in significant reductions in NE levels within the OB and impairment of the acquisition of the conditioned odour preference described above, suggesting a critical role for the LC in this type of learning (Sullivan *et al.*, 1994). The β-adrenoceptor agonist isoproterenol, admiinistered systemically, has been demonstrated to substitute for the effect of stroking in this paradigm, whereas β antagonist propranolol attenuates it (Langdon *et al.*, 1997; Sullivan *et al.*, 1991). It has been proposed that LC projections, acting upon β receptors in the OB, and glutamatergic input from the olfactory nerve, converge upon mitral cells in the OB to produce this conditioning (Yuan *et al.*, 2003). Accordingly, the paradigm has proven quite useful in the study of cellular and sub-cellular mechanisms of olfactory learning. Moreover, as glutamate and its various modulators comprise the predominant excitatory

neurotransmitter system throughout the human brain, findings resulting from this pursuit may be conducive to a more general model of human learning.

5-HT and NE Interacting as the Unconditioned Stimulus

At the cellular level, a temporal pairing of glutamate and β-adrenergic receptor activity in the OB appears necessary to induce odour preference learning. The effect of either stroking or isoproterenol, however, corresponds to dosage in an "inverted-U" fashion, such that there is an optimal dose that produces learning, which doses substantially above or below this optimum do not produce (Sullivan *et al.*, 1991; Yuan *et al.*, 2000). Stroking administered together with isoproterenol, moreover, lowers this optimal dose, suggesting that the effects of both are additive (Sullivan *et al.*, 1991). Serotonin (5-HT) receptor activation also appears to modulate this process, as rats pharmacologically depleted of bulbar 5-HT fibres require a higher optimal dose of isoproterenol to produce learning (Yuan *et al.*, 2000). Both administration of 5-$HT_{2A/2C}$ antagonist ritanserin and 5-HT fibre depletion attenuates approach learning behaviour of rat pups trained with stroking (McLean *et al.*, 1996). This deficit is restored by the application of DOI, a potent 5-$HT_{2A/2C}$ agonist. Serotonergic activity is not sufficient to produce learning without concurrent NE activity, however. More recent evidence suggests that NE β_1 and 5-HT_{2A} receptors colocalize in mitral cells of the OB, and that the synergistic activity of these receptors coregulates, in a linear fashion, the expression of cyclic adenosine monophosphate (cAMP; Yuan *et al.*, 2003), which is a ubiquitous second-messenger in the brain, and is strongly implicated in many models of synaptic plasticity and learning (e.g., Kandel *et al.*, 2000). cAMP is synthesized from AMP by

adenylyl cyclase, which can be activated by a variety of agents, including the G-protein-associated β_1 receptor, a phenomenon which is well documented.

Intracellular Signalling Systems Implicated in LTP

Further research has also implicated the involvement of cAMP response-element binding protein (CREB) as a critical component in some forms of long-term potentiation (LTP; Balschun *et al.*, 2003; Pittenger *et al.*, 2002) and long-term depression (LTD; Ahn *et al.*, 1999) of neuronal membranes. CREB is a nuclear regulatory transcription factor which, when activated through phosphorylation at its Ser133 residue, interacts with regulatory cAMP response elements (CREs) to promote the expression of a variety of downstream immediate-early genes (IEGs; reviewed in Bozon *et al.*, 2003). Manipulation of intracellular CREB levels is directly proportional to changes in both aversively- and appetitively-motivated memory formation (reviewed in Silva *et al.*, 1998). Quite recently, infusion of CREB antisense oligodeoxynucleotides (ODNs), which disrupt the transcription and synthesis of CREB, at 6 hr prior and during testing, but not 6 hr following, has been shown to attenuate aversive olfactory learning in rat pups (PND 11; Zhang *et al.*, 2003), suggesting that CREB is important in the acquisition of memory in this model.

Levels of phosphorylated CREB (pCREB) are transiently increased when odour and stroking are paired to produce learning in rat pups (PND 7). This increase, moreover, appears to occur specifically in regions of the OB activated by the peppermint odorant (McLean *et al.*, 1999). pCREB has also been shown to increase in response to isoproterenol paired with odour, but only at doses that produce learning. In parallel with

its inverted-U curve relationship to memory formation, the optimal dose of isoproterenol required to increase pCREB levels shifts to the right in bulbar 5-HT depleted rats (Yuan *et al.*, 2000). These findings appear to contrast with studies in *Aplysia*, which report increases in pCREB which correspond linearly to increases in cAMP levels, suggesting that the UCS converges on the CS upstream of cAMP formation in this paradigm, to ultimately activate CREB and its downstream effects (Kandel et al., 2000). In the present neonatal rat paradigm, cAMP, but not pCREB, is reportedly regulated in a linear fashion by β_1/5-HT_{2A} interaction. This suggests that the UCS converges on the CS downstream of cAMP in this model, and that effective cAMP activity is defined by a critical time window that is only satisfied by learning-effective interactions of β_1 and 5-HT_{2A} (Yuan *et al.*, 2000).

Signals Converging on Glutamate Receptors

Afferent stimulation from the olfactory nerve (ON) to mitral cells of the OB depends upon glutamate activity (Shipley *et al.*, 1996). Transient and/or lasting modification of the neuronal response to glutamate, therefore, is a putative mechanism of olfactory learning. In particular, the glutamate receptors specific for the ligands N-methyl-D-aspartate (NMDA) and α-amino-3-hydroxy-5-methyl-4-isoxazoleproppionate (AMPA), have been the subject of much investigation with respect to learning and memory paradigms. It was initially proposed that long-term changes to granule cell interneurons, which receive noradrenergic input, might result in disinhibition of the NMDA receptor at the dendrodendritic granule cell-mitral cell synapse, which might account for the formation of long-term memory (Sullivan and Wilson, 1994). In this

model, the UCS acts presynaptically to the mitral cell, and converges upon the CS through this disinhibition of glutamate receptors. Ca^{2+} transmission in granule cells is inhibited by NE and by α-adrenoceptor agonist clonidine, but not by isoproterenol, however (Trombley, 1992), suggesting that this model does not completely account for the results discussed above.

An alternative model focuses upon postsynaptic events within the mitral cell as a mechanism for olfactory learning. The NMDA receptor acts as a voltage-dependent ligand-gated ion channel, which is sufficiently large to channel Ca^{2+} ions when activated. Ca^{2+} influx has diverse effects within cell signalling systems; amongst these, it has been observed to affect both the levels of CREB phosphorylation (Mao & Wang, 2002; Vanhoutte *et al.*, 1999), and the rate and direction of synaptic plasticity (Shouval *et al.*, 2002). CREB can be activated by both cAMP-dependent protein kinase (PKA) and Ca^{2+}/calmodulin-dependent kinase IV (CaMKIV; Dash *et al.*, 1991), both of which pathways are influenced by intracellular Ca^{2+} levels (Waltereit & Weller, 2003). The functioning of NMDA itself can be modified through phosphorylation by a number of intracellular kinases, including PKA and PKC on its NR1 subunit (Seabold *et al.*, 2003; Tingley *et al.*, 1997), and CaMKII and SRC on its NR2B subunit (Soderling & Derkach, 2000). Only PKA, however, which phosphorylates the Ser897 residue of NR1 (Tingley *et al.*, 1997), has the distinction of being directly linked to β-adrenergic activity, which exerts such influence via the adenylyl cyclase to cAMP pathway. Robust activation of PKA, moreover, has been shown to occur during the induction of LTP in rat hippocampal slices, and such activation can be brought about by bath application of NMDA, which can also promote the generation of cAMP (Roberson & Sweatt, 1996). It is possible that, at

least to some extent, the CS and UCS converge to produce learning through activation of PKA.

Electrophysiological recordings from the rat OB indicate significant sensitization of both the NMDA and AMPA components of the ON-evoked field potential (ON-EFP) accompanying acquisition, but only in rats administered learning-effective doses of isoproterenol (Yuan *et al.*, 2000). This suggests that any critical time window for cAMP-induced activation of CREB should occur upstream of this sensitization of NMDA. cAMP acting via PKA might also exert an influence upon the AMPA receptor indirectly, by activating the enzyme inhibitor 1 (I1), which effectively inhibits the inactivation of CaMKII by protein phosphatase 1 (PP1; Blitzer *et al.*, 1998; Strack *et al.*, 1997). CaMKII is constitutively activated by Ca^{2+}/calmodulin, which stimulates autophosphorylation of its autoinhibitory domain (Braun & Schulman, 1995). Activated CaMKII rapidly associates with NMDA within the array of scaffolding proteins referred to as the postsynaptic density (PSD), and is at this point capable of enhancing AMPA channel conductance through phosphorylation of its GluR1 subunit – a phenomenon linked to early-phase LTP (McGlade-McCulloh *et al.*, 1993; Tan *et al.*, 1994). The increased AMPA conductance results in a more rapid depolarization of the membrane, which consequently weakens the Mg^{+} blockade of NMDA, and further enhances Ca^{2+} influx, in a positive feedback loop (Lisman, 2003; reviewed in Soderling & Derkach, 2000). Further supporting this line of reasoning are findings that AMPA receptor kinetics may be altered in response to induction of LTP through theta burst stimulation in hippocampus (Lin *et al.*, 2002). Taken together, these effects of PKA may at least

partially account for the convergence of signals which appears necessary for many forms of learning and memory, and odour approach preference learning in particular.

Is NR1 Phosphorylated by PKA in Olfactory Learning?

The present study seeks to elucidate the roles of these agents within the present olfactory learning paradigm. Firstly, it seeks to replicate the findings of Sullivan *et al.* and Yuan *et al.*, that rats given an optimal dose of isoproterenol (2mg/kg) paired with a peppermint odorant should demonstrate an approach preference for that odorant when tested 24 hr later. Rats that receive odour alone, isoproterenol alone, or neither, should not demonstrate such a preference. Secondly, if phosphorylation of NR1 by PKA is responsible for the alteration in the NMDA component of ON-EFPs, then such phosphorylation should occur at learning-effective doses of isoproterenol. This phosphorylation, moreover, should occur with such administration of isoproterenol both alone and paired with odour, particularly since odour was not required to produce an increase in ON-EFP in response to isoproterenol (Yuan *et al.*, 2000). PKA can be activated both through β_1 G-protein interactions and NMDA-mediated Ca^{2+} influx, either of which exerts its effect via the adenylyl cyclase cascade. However, the latter pathway, whose elevation of cAMP levels appears much more robust than that of β_1, has nonetheless been observed to be far less successful at inducing prolonged phosphorylation of the PKA-targeted Ser845 residue of GluR1 in rat hippocampal slices, and actually results in dephosphorylation of that site, possibly because NMDA is capable of activating various protein phosphatases as well as kinases (Vanhoose & Winder, 2003). Since PKA acting through the β_1 pathway appears to be more effective in this

manner, it is thus expected that both isoproterenol conditions should exhibit similar increases in pNR1, as indicted by Western blot analysis.

Materials and Methods

Animals

This study utilized a total of 64 Sprague-Dawley rat pups, from 8 litters. Dams and their litters were housed in polycarbonate cages (47 x 24 x 20 cm), at the Animal Care Unit of the Health Sciences Centre, Memorial University, on a 12 hour light/dark cycle. Treatment condition groups were comprised equally of male and female subjects. Cages were lined with wood chips and food and water were available ad libitum. All procedures were approved by the Memorial University Institutional Animal Care Committee, which adheres to guidelines set forth by the Canadian Council on Animal Care.

Assignment to Treatment Conditions

On post-natal day 6 (PND-6), an average litter weight was determined to establish the correct drug dosage per weight. Exceptionally small animals were excluded from the study. Eight pups were then numbered and assigned to one of the following four treatment conditions: Saline + No Odour (Naive, N), Saline + Peppermint Odour (Odour Only, O), 2 mg/kg Isoproterenol + No Odour (Iso Only, I), or 2 mg/kg Isoproterenol + Odour (Iso + Odour, I/O). This assignment was done randomly, except that one male and one female were assigned to each condition.

Drug Injections

On PND-6, rat pups were removed from their cages and administered 50 μl subcutaneous injections of either 2 mg/kg isoproterenol or saline, dependent upon their assigned treatment condition. Isoproterenol is a β-adrenergic receptor agonist, and a dosage of 2 mg/kg has been shown to mimic the effect of stroking in the odour conditioning paradigm (Sullivan *et al.*, 1994). Injections were administered 40 minutes prior to odour conditioning, to allow the drug to be most effective.

Odour Conditioning

On PND-6, the dam and litter were removed to the training room in the morning, and left for a number of hours to minimize any anxiogenic effects of transportation and the novel environment. Temperature in this room was maintained at 27^{o}C to keep pups warm when away from the dam. In the afternoon, pups were administered drug or saline injections, dependent upon their assigned treatment conditions, and returned to the dam. 30 min following injection, pups were removed from the nest and placed alone in bedding, for 10 min, and then placed in either peppermint-scented bedding (300 μl peppermint extract in 500 ml bedding), or unscented bedding, for a further 10 min. Peppermint training, as well as testing, occurred in a separate room to ensure prevent exposure of animals in non-odour treatment conditions (i.e., Naive or Iso). For each condition group, one of the subjects was then returned to the dam for testing 24 hours later, and the other was sacrificed 10 min later.

Odour Testing

The testing apparatus was a stainless steel test box (36 x 20 x 19 cm) with a square mesh bottom (1 x 1 cm grid). A finer polypropylene mesh (500 μm) was placed on the bottom of the box to facilitate locomotion of the subjects. Two wooden containers (18 x 18 cm), one containing peppermint-scented bedding (300 μl in 500 ml bedding), and the other containing unscented bedding, were placed beneath the test box such that a 1 cm "neutral" zone was left between them. Pups were placed in this neutral zone, and oriented in opposite directions for each subsequent test, to eliminate any direction preference bias. Testing occurred in five 1-min trials using this apparatus, and values were recorded for time spent over either scented or unscented bedding. A pup was considered to be over bedding if its nose and both forepaws were across the line defining the neutral zone.

Sacrificing

Four of eight pups (one for each treatment condition) were sacrificed by decapitation at 10 min following training, on PND-6. Olfactory bulbs were extracted from these pups, immediately frozen on dry ice, and stored in 1.5ml centrifuge tubes. Anterior olfactory nuclei were excluded from the sample by means of an angled transverse cut. Samples were stored at -70^{o}C to prevent protein degradation.

Western Blotting

Relative levels of the phosphorylated form of NR1 (pNR1) within the olfactory bulb were analyzed using a Western blotting technique. This technique, also referred to as sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (SDS-PAGE), is

commonly utilized in determining the relative protein content of samples, as specified by their particular molecular weights. Samples were removed from storage at -70^{o}C and immediately homogenized by application of a lysis buffer (0.1% SDS, 1% Nonidet P40, 20 mM Tris, 10% glycine, 1.37 mM with leupeptin [1mg/ml], apoprotin [8.9 U/ml], and sodium orthovanadate [1 mM]), and physical manipulation. The homogenized sample was then rotated at 4^{o}C for 30 min and centrifuged at 13,500 rpm for 15 min and only the lysate was retained.

The overall protein content of each lysate sample was then determined to ensure that an equal amount of protein was loaded in the subsequent SDS-PAGE procedure. This was accomplished by means of the Pierce BCA Protein Assay Kit (Rockford, Illinois), and a colour spectrophotometer. If not analyzed immediately, lysate samples were stored at –70^{o}C to prevent protein degradation.

Samples were separated by molecular weight using SDS-PAGE procedures. This study utilized a 4-20% gradient polyacrylamide gel, which allowed for better separation of the heavier proteins. pNR1, the protein of interest to this study, has a molecular weight of ~120 kDa, which is near the top of the scale of molecules detected by this apparatus (Bio-Rad Laboratories). Samples, in the appropriate amounts to yield 100μg protein, were added to 4 μl of 5X sample buffer (0.25 mM Tris-HCl, 10% SDS, 50% glycerol, 0.025% bromophenol blue, and 0.5% dithiothritol) and dH_20 was added to yield a total of 20 μl solution. This was micro-pipetted into wells within a 15% stacking gel. In addition, a colour-coded molecular weight standard solution (Bio-Rad) and a microsomal prep

positive control (Upstate Group) were included to facilitate identification of the band corresponding to pNR1 and NR1. Gels were immersed in a running buffer (25 mM Tris, 250 mM glycine, and 3.5 mM SDS, pH 8.3) and subjected to a current of 60 mA at 4^{o}C for ~80 min, or until the visible blue band had run past the bottom of the gel. Protein in the gels was then transferred onto a nitrocellulose membrane (Hybond ECL, Amersham), with immersion in a transfer buffer (25 nM Tris, 192 nM glycine, and 20% methanol) at 200 mA and 4^{o}C, for 1 hour.

Antibody Application

The presence of pNR1 was determined by application of a highly-specific antibody solution (primary antibody; 1/500, rabbit anti-phospho-NR1 [ser897], Upstate). The presence of NR1 was determined likewise (1/500, rabbit anti-NR1, Upstate). Following protein transfer, membranes were rinsed with phosphate-buffered saline plus Tween-20 (PBST), and a blocking agent (5% milk powder in PBST) was applied for 1 hour, to prevent non-specific binding of the antibody. The primary antibody was then applied overnight at 4^{o}C. The membrane was again rinsed with PBST (3 x 10 min) and a secondary antibody (1/2000, goat anti-rabbit IgG -HRP), specific for the primary and conjugated to horseradish peroxidase (HRP) for labelling, was applied for 1 hour with 2% milk powder. The presence of this antibody was detected by application of an HRP-binding chemiluminescence reagent (*Western Lightning, Perkin-Elmer*). The membrane was then immediately exposed to x-ray film and developed.

Analysis of Western Blot

Bands corresponding to pNR1 and NR1 were identified by reference to the molecular weight standard and the microsomal prep positive control. Once the correct band was identified, qualitative observations were made regarding the relative appearances of each treatment condition. Films were considered unanalyzable if (1) the pNR1 bands were obscured by excessive blotches, as probably introduced by the imperfect transfer of proteins from polyacrylamide gel to nitrocellulose membrane, (2) individual lanes were qualitatively observed to be darker relative to others when visualized with Ponceau stain, indicating that uneven amounts of total protein were originally loaded, or (3) other artifacts such as fading effects, possibly introduced by washing or antibody application, were observed on the film. In the latter case, antibodies were reapplied to the blots in an attempt to eliminate the artifact.

To obtain a quantitative measure of the relative amounts of bulbar pNR1 or NR1, films were analyzed using image analysis (ChemiImager, Alpha Innotech) to obtain an average pixel value (APV) for each band. This software utilized a liquid-cooled CCD camera to visualize the film, and then obtained an optical density measurement using greyscale pixel values (black = 255, white = 0). A region was drawn by the operator to represent each particular band (four for each film). The software then calculated an overall APV for the entire region. To minimize the effects of uneven background "noise" on the film, a background value was calculated for each region, derived from the 10 lowest pixel values, and this value was subtracted from the APV to obtain a new APV.

Results

Subjects did not learn in any treatment group.

None of the treatment groups exhibited a significant change in time spent over peppermint odour, as compared with the naive condition group; i.e., there was no main effect of treatment condition: one-way analysis of variance (ANOVA), $p > 0.05$. Figure 1 presents these data graphically. In particular, subjects receiving the Iso+Odour condition spent a mean of 43% of their time over peppermint-scented bedding when tested 24 hr after training, whereas the Naive group spent a mean of 29%. This result is inconsistent with numerous preceding reports (e.g., Sullivan et al., 1991; Langdon et al., 1997; Sullivan et al., 2000), in which subjects in the Iso+Odour condition spent a mean of ~60% of their time over peppermint, compared to a mean for the Naive condition that was similar to the present data, and this difference was significant.

pNR1 was significantly increased for Iso+Odour condition.

Average pixel values obtained from Western blotting techniques were significantly different across treatment conditions: repeated-measures ANOVA, $F(4, 19) = 4.567$, $p < 0.05$. These data consisted of blots obtained from five separate trials, performed on five separate litters. Blots for the remaining three trials did not yield analyzable films (see Materials and Methods) and were excluded on this basis. A post-hoc Tukey-Kramer multiple comparisons test revealed a significant difference between the Naive and Iso+Odour conditions, indicating an increase in pNR1 for the latter condition: $q = 4.798$, $p < 0.05$. Differences between other groups were not significant;

however, as presented graphically in Figure 2, there was a non-significant trend proceeding from Naive to Odour to Iso to Iso+Odour conditions. Average pixel values for NR1 were subsequently obtained from three of the blots initially analyzed for pNR1. Analysis of these values indicated no significant main effect of treatment conditions: repeated measures ANOVA, $p > 0.05$. This indicates that the increase observed in pNR1 is not due to an increase in total NR1 (Figure 2).

In a few cases, the band corresponding to an individual treatment condition was partially obscured by blotching effects. This band was analyzed by selecting the unobscured region with the imaging software, carefully avoiding any portion potentially affected by the blotching artifact. This process yielded results that agreed with qualitative observations, and the obtained data were included in the analysis on this basis.

Discussion

Possible Sources of Error for Behavioural Results

None of the treatment groups exhibited a significant difference from controls in odour approach preference behaviour (Figure 1). These results contrast with many preceding reports, including some that have utilized the same facilities and apparatus as the present study, that a 2mg/kg dosage of isoproterenol paired with peppermint odour produces a behavioural preference when tested 24 hr later (e.g., Langdon et al., 1997; Sullivan et al., 1991). These conflicting reports suggest that the lack of significance in the present study is due to confounding factors, or procedural error, or a combination of these. A possible factor that was difficult to control for was the anxiety of the dam, which could only be estimated through casual observation of behaviour, and varied noticeably between litters. Litters were removed to training rooms early in the morning, and trained in the afternoon in an attempt to minimize this factor. Nonetheless, distress of the dam, which can conceivably be easily transferred to her pups, particularly if she handles them roughly, remained evident throughout the study. Emotional stress can critically disrupt the neural systems involved in this learning model, particularly by activation of the LC. Injection technique was another matter of concern in this respect, as improper technique can result in administering an incomplete dosage, or misdirecting drug delivery (e.g., into an organ rather than subcutaneously), which can alter the intended acuity and/or latency of the drug effect. Had this occurred, however, one should not expect to find an increase in pNR1, assuming this depends upon an optimal dose of isoproterenol, as is

hypothesized. Such an effect was indeed found in the present study, suggesting against the possibility that isoproterenol was improperly injected.

Phosphorylation of NR1 in the Iso+Odour Condition

Despite the failure to replicate the odour conditioning of previous studies, the present study did find a significant increase in pNR1 for the Iso+Odour group, as compared to the Naive control condition. This finding is important because it supports electrophysiological evidence of a sensitization of the NMDA component of ON-EFPs, which is found only at learning-effective doses of isoproterenol (Yuan et al., 2000). It also introduces many new questions. Since the Ser897 residue of NR1 is specifically phosphorylated by PKA (Tingley et al., 1997), introduction of an appropriate PKA inhibitor such as H89 should attenuate the result found here. It would be interesting to test whether non-optimal doses of isoproterenol will result in phosphorylation of this site, as ON-EFP results suggest that they will not. Moreover, if a critical time window exists for the induction of learning via cAMP, as is suggested by the emerging model, this might be elucidated by further evaluating the role of the various protein phosphatases activated both by cAMP and NMDA pathways. It seems prudent to speculate that an intricate equilibrium existing between the opposing effects of these enzymes, and subtle disruption of this equilibrium by ongoing extracellular events and the receptor molecules that transduce them into intracellular signals, is responsible for such a window leading ultimately to long term cellular memories.

Effects of β_1 and NMDA Interactions

Whether the non-significant trend from Naive to Odour to Iso to Iso+Odour, apparent in Figure 2, might be supported by a more powerful research design is an open question. That the Iso condition in particular is not significantly greater than controls is contrary to the initial hypothesis, but it is possible that odour, activating NMDA through the ON, does play a critical role in NR1 phosphorylation. Bath application of NMDA (a ligand that activates the NMDA-type glutamate receptor) to hippocampal CA1 slices is sufficient to activate PKA (Roberson & Sweatt, 1996), and also causes a increase in cAMP generation which is far more robust than that observed with isoproterenol (Vanhoose & Winder, 2003). However, as noted above, this same NMDA treatment fails to phosphorylate Ser845 on GluR1, and actually dephosphorylates this residue, whereas isoproterenol induces a prolonged phosphorylation at this site. NMDA, moreover, stimulates the activation of a number of protein phosphatases which, when concurrently blocked, eliminate the dephosphorylation, but do not promote phosphorylation, at Ser845 (Vanhoose & Winder, 2003). This evidence suggests that PKA-induced phosphorylation of the Ser897 site on NR1, if it behaves similarly, should be *decreased* by pairing with odour. However, there is no reason to believe that phosphorylation on these sites should behave similarly, other than their observed specificity for PKA, and it is likely that factors particular to PKA-GluR1 interactions, as well as spatial localization of Ca^{2+}-specific adenylyl cyclases, mediate the phenomenon reported in this study (discussed in Vanhoose & Winder, 2003). Further research focusing specifically on interactions between β_1 and NMDA and their effects on NR1 phosphorylation within the present olfactory learning paradigm are necessary to better explain the trend noted in Figure 2.

Involvement of AMPA Receptors

In the same hippocampal study, NMDA does result in significant phosphorylation of GluR1 at Ser831, which is targeted by both CaMKII and PKC (Vanhoose & Winder, 2003). Dephosphorylation of Ser845, as is seen in response to NMDA applied alone in this study, has elsewhere been linked to the production of LTD (Kameyama et al., 1998). The application of isoproterenol immediately prior to NMDA eliminates the dephosphorylating effect of the latter, whereas the reverse application does not (Vanhoose & Winder, 2003). It is possible that varying levels of β_1-adrenergic activation, moderated by 5-HTT activation, and initiating immediately prior to NMDA activation, are capable of adjusting the phosphorylation state of Ser845 accordingly, essentially "fine-tuning" the less-specific activation of Ser831 to determine the direction of plasticity. By applying the appropriate constraints, this possibility might be tested within the present olfactory learning paradigm.

In addition to this NMDA-modulated phosphorylation of AMPA, there is also increasing evidence to support a "silent synapse" model for the postsynaptic glutamate response. In silent synapses, functional AMPA receptors are effectively absent from the postsynaptic membrane, and NMDA activation results in a rapid recruitment of these receptors to membrane-bound positions, which phenomenon has been observed to occur in hippocampal slices within minutes of excitation (Liao *et al.*, 2001). This is paralleled by a significant increase in AMPA receptor-mediated miniature excitatory postsynaptic currents (mEPSCs). Whether or not a synapse is silent, however, it is evident that lasting alterations to the expression of AMPA receptors, and their structural organization, is involved in the production of LTP. It is possible that activation of CREB by the processes

described herein result in increased transcription and synthesis of AMPA receptor proteins. By parallel processes, vesicular GluR1 and GluR2 are possibly differentially "trafficked" to dendritic sites proximate to excitatory stimuli, and further alterations to the organization of PSD proteins, to accommodate an increase in membrane-expressed AMPA receptors, may underlie long-term increases in synaptic sensitivity. These processes appear to be regulated to a large extent by the activity of constitutively active CaMKII (reviewed in Lisman, 2003).

Conclusions

The present findings help define an emerging model of olfactory learning that has exciting implications for the study of learning and memory in general. It opens the door to further investigation of the specific changes to NMDA and AMPA receptors in particular, and the temporal pattern of these changes corresponding to long-term synaptic plasticity. Research might focus on the phosphorylation of other sites on these receptors, for instance, as well as the kinases and phosphatases that directly affect phosphorylation state. An exhaustive investigation of this sort might greatly clarify the nature of the relationship of cAMP levels to the activation of CREB, and the putative critical time window for cAMP activity (Yuan et al., 2000). The role of NE/5-HT interaction in "fine-tuning" glutamate and Ca^{2+} activity, and the importance of this to the production and direction of long-term plasticity has also yet to be fully understood, and might be investigated in a similar manner. In addition to this postsynaptic model, presynaptic disinhibition of granule cells by NE may also play an important role in the formation of olfactory learning, which is neither supported nor refuted by the present study.

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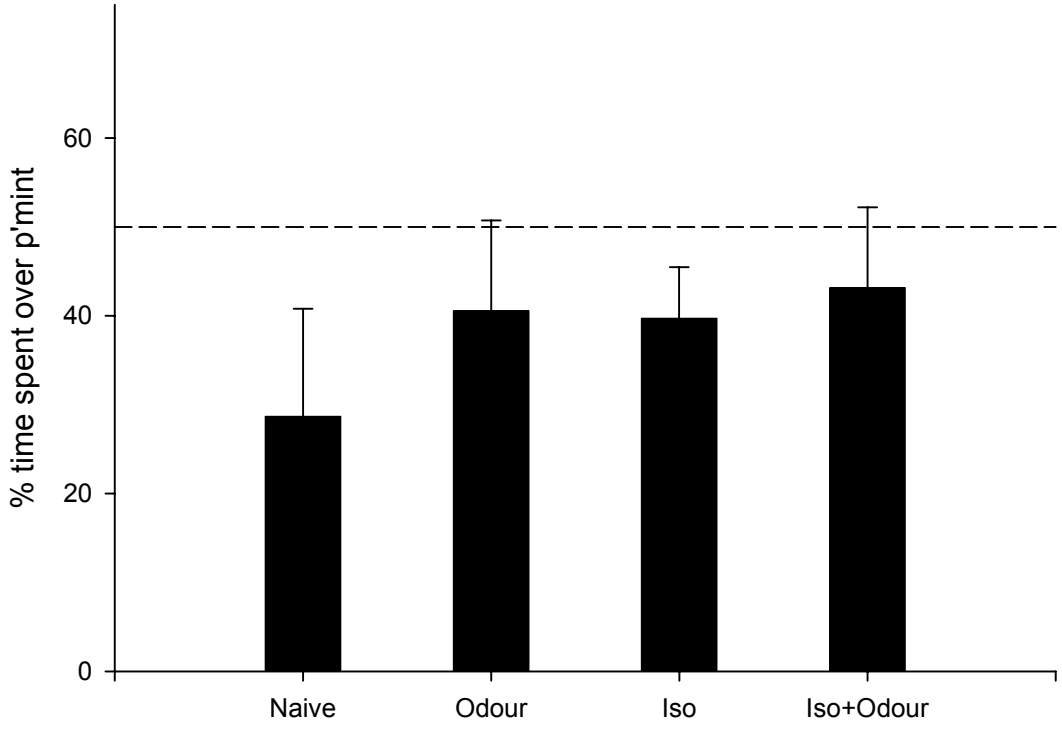

Figure 1. *None of the treatment groups exhibited a significant difference from the Naive condition in the percentage of time spent over peppermint-scented bedding versus unscented bedding: (ANOVA), p>0.05. Dashed line is 50%. Bars represent standard error of the mean (SEM).*

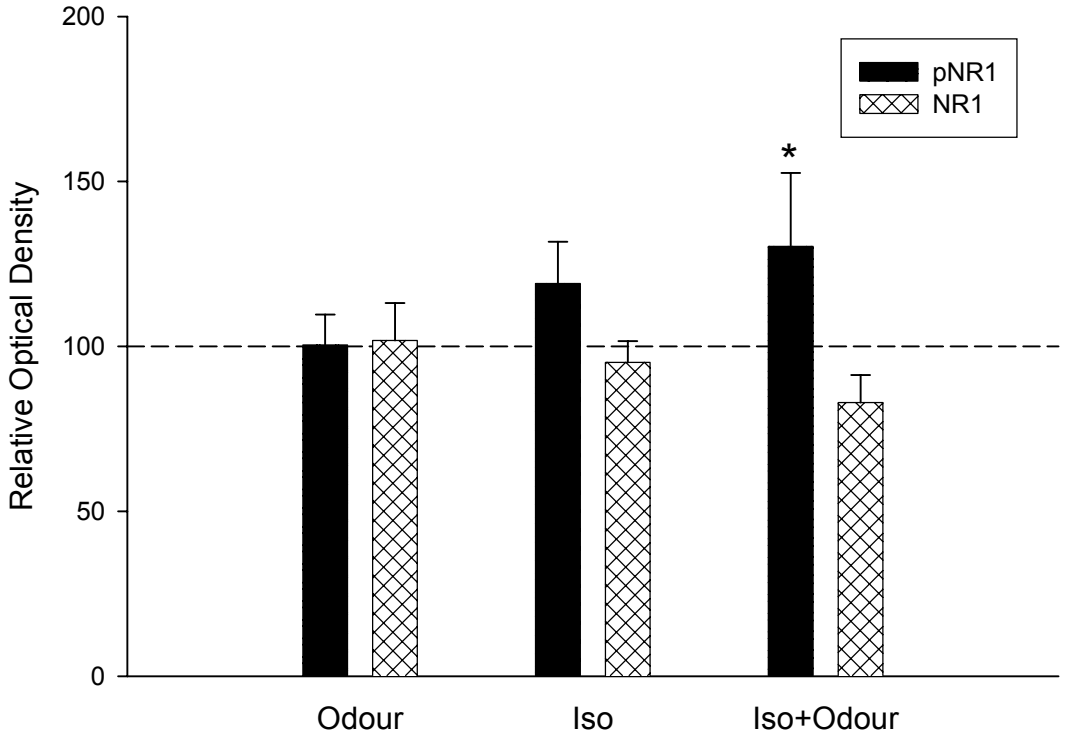

Figure 2. *Optical densities (average pixel values, relative to Naive condition within trials) for pNR1 and NR1. The main effect of treatment conditions for pNR1 was significant: repeated measures ANOVA, $F(4, 19) = 4.567$, $p < 0.05$, $N = 5$. The Iso+Odour condition differed significantly from Naive controls: Tukey-Kramer test, $p < 0.05$ (*). This indicates a significant increase in pNR1 for this condition. There was no significant main effect for treatment conditions for NR1, indicating that increases in pNR1 were not due to increases in total NR1: repeated measures ANOVA, $p > 0.05$, $N = 3$. Dashed line represents 100% of Naive values. Bars represent standard error of the mean (SEM).*

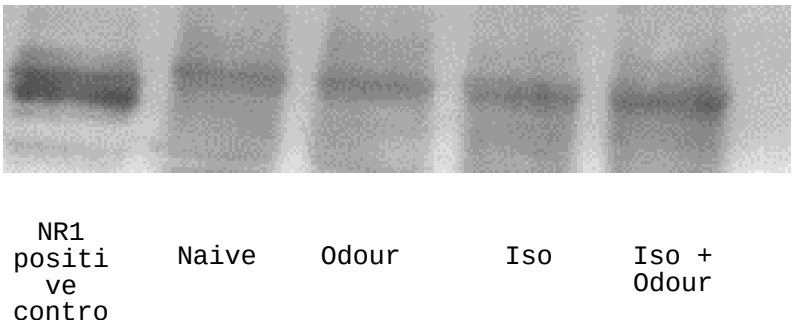

Figure 3. *Image of Western blot for pNR1, labelled for treatment condition.*