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NAAG peptidase inhibitors and deletion of NAAG peptidase gene enhance memory in novel object recognition test

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Abstract

The peptide neurotransmitter N-acetylaspartylglutamate (NAAG) is inactivated by the extracellular enzyme glutamate carboxypeptidase II. Inhibitors of this enzyme reverse dizocilpine (MK-801)-induced impairment of short-term memory in the novel object recognition test. The objective of this study was to test the hypothesis that NAAG peptidase inhibition enhances the long-term (24 hr delay) memory of C57BL mice in this test. These mice and mice in which glutamate carboxypeptidase II had been knocked out were presented with two identical objects to explore for 10 minutes on day 1 and tested with one of these familiar objects and one novel object on day 2. Memory was assessed as the degree to which the mice recalled the familiar object and explored the novel object to a greater extent on day 2. Uninjected mice or mice injected with saline prior to the acquisition session on day 1 demonstrated a lack of memory of the acquisition experience by exploring the familiar and novel objects to the same extent on day 2. Mice treated with glutamate carboxypeptidase II inhibitors ZJ43 or 2-PMPA prior to the acquisition trial explored the novel object significantly more time than the familiar object on day 2. Consistent with these results, mice in which glutamate carboxypeptidase II had been knocked out distinguished the novel from the familiar object on day 2 while their heterozygous colony mates did not. Inhibition of glutamate carboxypeptidase II enhances recognition memory, a therapeutic action that might be useful in treatment of memory deficits related to age and neurological disorders.

Keywords

N-Acetylaspartylglutamate; NAAG; memory; glutamate carboxypeptidase II knockout mice; mGluR3

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1. Introduction

Heterotropic agonists of the group II metabotropic glutamate receptors, mGluR2 and mGluR3 have been reported to have negative or neutral outcomes in animal models of cognition (Gravius et al., 2010; Marek, 2010). For example, the mGluR2/3 agonist LY354740 impaired attention and working memory (Aultman and Moghaddam, 2001; Higgins et al., 2004; Schlumberger et al., 2009; Spinelli et al., 2005) but failed to induce detectable cognitive impairments in healthy volunteers (Dunayevich et al., 2008; Krystal et al., 2005). The group II agonist DCGIV impaired learning and memory of a passive avoidance task in mice apparently via the group II receptors negative coupling to adenylate cyclase (Sato et al., 2004). In contrast to these data on the neutral or anti-cognitive effects of group II mGluR agonists, the mGluR2 positive allosteric modulator LY487379 is reported to increase cognitive flexibility in rats (Nikiforuk et al., 2010).

Interpretation of these data is complicated by the use of agonists and antagonists that interact with both mGluR2 and mGluR3 *in vitro* and *in vivo* (Kingston et al., 1998; Linden et al., 2009; Monn et al., 1999; Rorick-Kehn et al., 2007). LY354740 induced cognitive impairment in the Morris Water Maze in wild type mice but not mGluR2 knockout mice, leading to the conclusion that this effect was mediated via mGluR2 (Higgins et al., 2004). In animal models of schizophrenia, the effects of this agonist are similarly absent in mGluR2 while present in mGluR3 knockout mice (Linden et al., 2009; Rorick-Kehn et al., 2007).

In contrast, the peptide neurotransmitter N-acetylaspartylglutamate (NAAG) is a selective mGluR3 agonist (Neale, 2011; Olszewski et al., 2012a). Inhibitors of glutamate carboxypeptidase II (GCP II), the enzyme that inactivates NAAG, elevate extracellular levels of the peptide and increase activation of this receptor (Adedoyin et al., 2010; Slusher et al., 1999; Zhong et al., 2006; Zuo et al., 2012). NAAG peptidase inhibitors are effective in animal models of several clinical conditions (Neale et al., 2005; 2011; Thomas et al., 2006; Wozniak et al., 2012) and rescue short-term memory impairment induced by a low dose of dizocilpine (MK801) (Olszewski et al., 2012b). This latter result suggested that these inhibitors also might affect learning or memory in mice in which cognition had not been artificially diminished. The aim of this study was to determine if NAAG peptidase inhibitors affected long-term memory in the novel object recognition test in C57BL mice.

2. Methods

2.1. Animals

The experimental protocols used in this research were approved by the Georgetown University Animal Care and Use Committee consistent with guidelines of the US National Institutes of Health. Seven to 11 week old adult male C57BL/6NCr mice were from the National Cancer Institute, Frederick Research Center. Two glutamate carboxypeptidase II knockout males (Bacich et al., 2002) were provided by Warren Heston, rederived by IVF in Jackson Laboratory (Bar Harbor, ME) and ten pathogen free mice (four females and six males) were transferred to Georgetown where a colony was established. The knockout mice used in this study were backcrossed at least ten times to C57BL/6NCr. Heterozygous knock out mice expressed about 50% less GCP II protein and significantly less NAAG hydrolase activity than did wild type littermates (Bacich et al., 2002). Mice were housed 5 to a cage and maintained on a 12:12 h light-dark cycle with food and water available *ad libitum*. Behavioral testing was performed during the light cycle between 10 am and 4 pm.

2.2. Drugs

The GCP II/NAAG peptidase inhibitor ZJ43 (N-[[[(1S)-1-Carboxy-3-methylbutyl]amino]carbonyl]-L-glutamic acid) was synthesized as previously described

(Olszewski et al., 2004) and provided by Alan Kozikowski. LY341495 ((2S)-2-Amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid), a selective group II mGluR antagonist (Kingston et al., 1998), LY354740 ((1S,2S,5R,6S)-2-Aminobicyclo[3.1.0]hexane-2,6-dicarboxylic acid), a heterotropic group II mGluR agonist (Monn et al., 1999), and 2-PMPA (2-(Phosphonomethyl)pentane-1,5-dioic acid), another potent GCPH inhibitor (Jackson and Slusher, 2001; Tsukamoto et al., 2007), were from Tocris Cookson Ltd. (Bristol, UK). All compounds were dissolved in saline and injected i.p.

2.3. Novel Object Recognition Test

Novel object recognition is a validated and widely used test for assessing recognition memory (Antunes and Biala, 2011; Akkerman et al., 2012; Lyon et al., 2012; Zhang et al., 2012). Mice were placed individually in a 22 × 32 × 30 cm testing chamber with beige walls for a 5 min habituation interval followed by injection with saline or ZJ43 and returned to home cage. Thirty minutes later mice were placed in the testing chamber for 10 min with two identical objects (acquisition session). Mice were returned to home cages and one day later placed back into the testing chamber in the presence of one of the original objects and one novel object (recognition session) for 10 minutes. The original objects consisted of two smooth surfaced weighted red cylinders 7 cm high × 4 cm diameter at base. The novel object consisted of a blue, 7 cm high × 5 cm diameter (base) round pyramid. The acquisition and recognition sessions were video recorded and an observer who was blinded to drug treatment scored the time spent exploring the objects. The chambers and objects were cleaned with ethanol between trials. Exploratory behavior was defined as sniffing, touching and directing attention to the object. In preliminary studies, naive mice exhibited no significant preference for the red cylinder or the blue pyramid. Exploration time (Table 1) is expressed as the mean ± the standard error of the mean (S.E.M.). For the acquisition session, the recognition index (RI) was calculated as (time exploring one of the objects/the time exploring both objects) × 100. For the recognition session, the RI was calculated as (time exploring the novel object/the time exploring both the familiar and novel object) × 100. The discrimination ratio for the retention trial on day 2 was calculated as the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in recognition session on day 2 (Ennaceur and Delacour, 1988).

2.4. Statistical Analysis

For the novel object recognition test, the time spent exploring each object was analyzed by two-way repeated measures ANOVA, with session as within-subject factor and treatment as a between-subject factor. Discrimination ratio data were analyzed by one-way ANOVA followed by Student-Newman-Keuls post-hoc test.

3. Results

3.1. Total exploration times

The time exploring individual objects during acquisition trials and recognition trials for each treatment group and the discrimination ratios for the recognition session are presented in Table 1. Within ZJ43 and 2-PMPA treatment groups, there was a wide range of total exploration times in the acquisition session. The exploration times of the three groups of saline treated mice were within a narrower range while uninjected mice had the highest level of exploration times during this session. In contrast to the group II agonist LY354740, treatment with the most effective dose of ZJ43 (150 mg/kg) did not significantly affect total exploration times during the acquisition session relative to the saline control groups.

3.2. Effects of NAAG peptidase inhibitors ZJ43 and 2-PMPA

Statistical analyses of the efficacy of the NAAG peptidase (GCPII) inhibitor ZJ43 (Fig. 1) revealed a significant effect of drug ($F_{(6,58)} = 22.09$, $P < 0.001$), session ($F_{(1,58)} = 136.36$, $P < 0.001$), and drug x session interaction ($F_{(6,58)} = 11.75$, $P < 0.001$). Control C57BL mice injected with saline or given no injection demonstrated no memory in the 24 hr delay novel object recognition test, exploring the identical objects about 50% of the time during the acquisition session and similarly exploring the familiar and novel objects about the same amount of time during the recognition session (Table 1). In contrast, mice treated with 100 and 150 mg/kg ZJ43 explored the novel object significantly more time than the familiar object during the recognition session ($P < 0.001$). The procognitive effect of ZJ43 (150 mg/kg) was significantly reduced by co-administration of the mGluR2/3 antagonist LY341495 ('LY95' in table and figures, 2 mg/kg) consistent with NAAG activation of mGluR3 ($P < 0.05$ for ZJ43 vs. ZJ43+LY95). The mGluR2/3 agonist, LY354740 (10 mg/kg) significantly increased the time spent attending the novel object versus the saline treated mice ($P < 0.001$).

In the study of the effects of the NAAG peptidase (GCPII) inhibitor 2-PMPA (Fig. 2), there was a significant main effect of drug ($F_{(6,57)} = 7.46$, $P < 0.001$), session ($F_{(1,57)} = 109.46$, $P < 0.001$) and interaction between session and drug ($F_{(6,57)} = 4.34$, $P < 0.001$). 2-PMPA significantly enhanced exploration of the novel object during the retention trials at 10, 50, 100 mg/kg ($P < 0.001$ for all three groups). Again the mGluR2/3 antagonist LY341495 (LY95) reduced the effects of peptidase inhibitor (2-PMPA, 100 mg/kg versus 2-PMPA with LY341495 ($P < 0.01$)). When 2-PMPA (100 mg/kg) was administered 10 minutes after the acquisition trial, exploration of the novel object on day 2 was not significantly different from the saline treatment group ($P = 0.8$), and significantly lower than when the same dose of 2-PMPA was administered 30 min before the acquisition session ($P < 0.05$).

3.3. GCPII knockout mice

Mice in which GCPII had been knocked out, were tested for memory in the 24 hr delay novel object recognition test (Fig. 3). There was significant effect of genotype ($F_{(1,22)} = 7.11$, $P < 0.05$) and session ($F_{(1,22)} = 47.66$, $p < 0.001$). Heterozygous mice treated with saline (HET/S) failed to reveal memory of the acquisition session and explored the novel and familiar objects to a similar extent during the recognition session on day 2. In contrast, mice from the same colony that lacked GCPII explored the novel object significantly longer than the familiar object on day 2 ($P < 0.001$). Like wild type C57BL mice, heterozygous GCPII mice treated with 2-PMPA (100 mg/kg) spend significantly more time exploring the novel object than the familiar object during the recognition session ($P < 0.001$). There was no significant difference between the knockout mice treated with saline or treated with 2-PMPA ($P = 0.8$).

4. Discussion

4.1. Acquisition or Retention

The data presented here demonstrate that NAAG peptidase inhibition positively affects object recognition memory as tested in the 24 hr delay novel object recognition test. These mice have a high level of recall when tested for novel object recognition 1.5 hr after acquisition (Olszewski et al., 2012b). In the 1.5 hr delay test, 2-PMPA reverses memory deficits elicited by a low dose of the NMDA antagonist dizocilpine when given before but not after the acquisition trial. Similarly, when 2-PMPA was given immediately after the acquisition trial in the present study, it was significantly less effective in improving retention than when administered just prior to the acquisition session. Taken together these data suggest that NAAG peptidase inhibition enhances acquisition but may also have a role in consolidation immediately after the training trial. In the only previous report in which the cognitive effects of NAAG peptidase inhibition was tested, 2-PMPA (50 and 100 mg/kg)

administered prior to acquisition training in a passive avoidance task did not significantly enhance long-term memory in rats assessed 24 hrs after acquisition while 150 mg/kg impaired alternation behavior (Lukawski et al., 2008). However, positive effects could not be assessed in this study since latencies were not recorded above 180s and control and drug treated mice reached this ceiling.

4.2. Mechanism of action of ZJ43 and 2-PMPA

The efficacy of two the structurally different drugs ZJ43 and 2-PMPA, neither of which acts as an agonist or antagonist at group II mGluRs (Yamamoto et al., 2004; 2007), is consistent with the current understanding of their mechanism of action via inhibition of glutamate carboxypeptidase II and the consequent elevation of synaptic NAAG levels (Neale et al., 2011; Slusher et al., 1999; Zhong et al., 2006; Zuo et al., 2012). The performance of the glutamate carboxypeptidase knockout mice on the novel object recognition test supports this model of NAAG function as does the reduced efficacy of ZJ43 and 2-PMPA in the presence of the group II mGluR antagonist LY341495 and the efficacy of the group II agonist LY354740 in replicating the effect of the peptidase inhibitors. The failure of 2 mg/kg of LY341495 to fully block the effect of NAAG peptidase inhibition and of a 3 mg/kg dose to further reduce the effect of ZJ43 (data not shown) and the diverse cognitive effects of this and another group II antagonist on memory (Pitsikas et al., 2012; Sato et al., 2004; Shimazaki et al., 2007) may well be related to this antagonist's activity at mGluR2 and other receptors (Kingston et al., 1998; Linden et al., 2009; Pitsikas et al., 2012; Sato et al., 2004).

Additionally important, the replication of the effect of NAAG peptidase inhibition in the colony of mice that were heterozygous for GCP2 (Fig. 3) represents a confirmation of the effect observed in the inbred C57BL/6J mice (Fig. 1 and 2). These GCP2 heterozygous mice have significantly lower levels of GCP2 expression and NAAG peptidase activity (Bacich et al., 2002).

4.3. Heterotropic group II mGluR agonists vs. NAAG peptidase inhibitors

This is the first report of a procognitive effect of a group II mGluR agonist in novel object recognition outside of models of pathological conditions. These data are most similar to the efficacy of the mGluR2/3 agonist LY379268 in improving novel object recognition in mice raised in social isolation (Jones et al., 2010). The cognitive effects of heterotropic group II mGluR agonists appear dependent on the species tested and the test used. LY354740 and a positive allosteric modulator of mGluR2 improved PCP- and ketamine-induced deficits in working memory and cognition in rodents and healthy human subjects (Boyle et al., 2004; Harich et al., 2007; Krystal et al., 2005; Moghaddam and Adams, 1998). While group II mGluR agonists and positive allosteric modulators are in different stages of preclinical studies and clinical trials as therapies for cognitive deficits observed in schizophrenia (Gregory et al., 2011; Kinon et al., 2011; Plath et al., 2011), none have been reported to enhance memory when tested alone in healthy volunteers. With the exception of the mGluR2 positive allosteric modulator LY487379 increasing cognitive flexibility (Nikiforuk et al., 2010), there are no previous reports that mGluR2/3 agonists have positive effects on memory outside of animal models of schizophrenia. Rather, several reports indicate that the heterotropic mGluR2/3 agonist LY354740 impairs rather than enhances attention and working memory (Aultman and Moghaddam, 2001; Higgins et al., 2004; Schlumberger et al., 2009; Spinelli et al., 2005).

Studies in knockout mice have demonstrated that of the behavioral effects of heterotropic group II mGluR agonists are mediated by mGluR2 in animal models of schizophrenia (Fell et al., 2008; Woolley et al., 2009). Similarly, LY354740 induced cognitive impairment in the Morris Water Maze in mGluR3 but not mGluR2 knockout mice (Higgins et al., 2004). In

contrast to these group II agonists, the behavioral effects of NAAG peptidase inhibitors are mediated by mGluR3 (Neale, 2011; Olszewski et al., 2012a).

4.4 Clinical relevance of Novel Object Recognition Test

Neuronal pathways mediating cognition are complex and vary based on the cognitive process being executed and the test or condition being studied. The novel object recognition test (Ennaceur and Delacour, 1988) is often used to assess visual learning and recognition processing (Antunes and Biala, 2011; Floresco and Jentsch, 2011; Young et al., 2009) and is similar to the visual paired-comparison task in humans (Clark et al., 2000; Manns et al., 2000). As a result, it is used to test recognition memory in animal models of clinical disorders including ischemia (Dhawan et al., 2012; Pazos et al., 2012), Alzheimer's disease (Greco et al., 2010; Lykhmus et al., 2011; Taglialatela et al., 2009; Zhang et al., 2012), schizophrenia (Lyon et al., 2012), Huntington's disease (Giralt et al., 2011) and ADHD (Levin et al., 2011). Recognition memory for novel objects also serves as a marker for clinical diagnosis of Alzheimer's (Lee et al., 2003). However, 24 hr discrimination in the novel object recognition test represents a single type of cognitive assessment and within this test there appear to be species/strain specific variation in the baseline recognition. For example in contrast to C57BL mice, the DA rat strain demonstrate significant discrimination 24 hr after acquisition (Barker et al., 2006). Given this and the nature of the memory tested by novel object recognition, it will be important to expand assessment of these NAAG peptidase inhibitors in behavioral studies that more broadly assess their potential procognitive effects.

4.5. Conclusion

There are a variety of small molecule targets being studied with the aim of enhancing cognition (Plath et al., 2011). These include acetylcholine and metabotropic glutamate receptor agonists, histamine and serotonin antagonists and phosphodiesterase inhibitors. The most widely used cognition enhancing drugs are cholinesterase inhibitors and the NMDA receptor antagonist memantine for Alzheimer's Disorder, modafinil (off label) for schizophrenia, and psychostimulants like methylphenidate for ADHD. While most appear to affect attention, they have limited impact on complex cognitive processing and memory. As a result, there is a need to develop new lines of cognition enhancing drugs with different targets. The data presented here suggest that NAAG peptidase inhibition and NAAG activation of mGluR3 represents one such novel approach to memory enhancement that warrants further study.

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Mice treated with saline (n = 13) prior to the acquisition session on day 1, explored the novel object and familiar object about equal amounts of time on the recognition session on day 2. ZJ43 (100 and 150 mg/kg, n = 13 and 9, respectively) increased novel object recognition on day 2. The group II mGluR agonist LY354740 (LY40, 10 mg/kg, n = 6) similarly increased recognition of the novel object. The group II mGluR antagonist LY341495 (LY95, 2 mg/kg) coinjected with 150 mg/kg ZJ43 (n=10) significantly reduced the efficacy of ZJ43 ($P<0.05$). Recognition index for the acquisition session = (the time exploring one object/time exploring both objects) $\times$ 100 and for the recognition session = (time exploring the novel object/time exploring both objects) $\times$ 100. FO(1) = familiar object 1; FO(2) = familiar object 2 (these objects are identical in shape, size and color); NO = novel object which differs in shape and color from the familiar objects. In this and the following figures: * $P<0.05$; ** $P<0.01$; *** $P<0.001$. 2-PMPA increased novel object recognition in a dose dependent manner (n = 9, 6, 10, 9 for 0.2, 10, 50 and 100 mg/kg treatment groups respectively). Coinjection of 100 mg/kg 2-PMPA with 2 mg/kg LY343495 (LY95, n=10) significantly reduced novel object recognition relative to treatment with 100 mg/kg 2-PMPA alone ($P<0.01$). Mice treated with 100 mg/kg 2-PMPA 10 minutes after the acquisition session (POST-AS, n=6) did not differ significantly from saline treated group ($P=0.8$) but this dose of 2-PMPA significantly decreased recognition when compared with the same treatment (100 mg/kg) administered 30 minutes prior to the acquisition session ($P<0.05$). Data for saline treated mice are the same as presented in Fig. 1. Saline treated mice heterozygous (HET/S, n = 7) for glutamate carboxypeptidase II (GCPII, NAAG peptidase inhibitor) failed to distinguish the novel from the familiar object when tested on day 2. In contrast, the GCPII knockout mice (GCPII KO/S, n = 6) spent significantly more time exploring the novel than the familiar object during the recognition session (HET/S vs GCPII KO/S, $P<0.05$). Treatment with 100 mg/kg 2-PMPA increased recognition of the novel object on day 2 for the GCPII heterozygous mice (HET/2-PMPA, n = 7), (HET/S vs. HET/2-PMPA, $P<0.01$). 2-PMPA had no significant effect on GCPII knockout mice in this assay. The same HET and GCPII knockout mice were tested with saline and with 2-PMPA in this experiment. Saline treatment was given first followed by 2-PMPA one week later.

Fig. 1.
Effect of NAAG peptidase inhibitor ZJ43 on novel object recognition

Fig. 2.
Effect of NAAG peptidase inhibitor 2-PMPA on novel object recognition

Fig. 3.
Novel Object Recognition by GCPII knock-out mice.

Exploration time for each group expressed as the mean $\pm$ the standard error of the mean (S.E.M.). The discrimination ratio for the retention trial on day 2 was calculated as the difference in exploration time expressed as a proportion of the total time spent exploring the two objects in recognition session on day 2.

Table 1

Group	Genotype/Injection	Acquisition session [sec.]			Recognition session after 24 hrs [sec.]			Discrimination Ratio
		N	FO (1)	FO (2)	NO	FO (2)		
1	no injection	10	23 (2.5)	24 (2.6)	17 (2.5)	16 (1.5)		-0.02 (0.07)
2	saline	13	16 (2.9)	16 (2.8)	19 (2.3)	17 (2.3)		0.07 (0.04)
3	ZJ43 (50 mg/kg)	10	11 (1.6)	11 (1.5)	17 (1.7)	13 (1.6)		0.16 (0.05)
4	ZJ43 (100 mg/kg)	13	14 (1.7)	12 (1.2)	33 (3.0)	10 (1.7)		0.57 (0.05)
5	ZJ43 (150 mg/kg)	9	20 (3.6)	20 (3.9)	38 (7.2)	13 (2.6)		0.51 (0.05)
6	LY95 (2 mg/kg)	10	20 (2.4)	18 (2.3)	26 (2.3)	30 (2.1)		-0.07 (0.03)
7	LY95 (2 mg/kg)+ZJ43(150 mg/kg)	10	10 (1.6)	9 (1.6)	26 (3.3)	14 (2.5)		0.31 (0.04)
8	LY40 (10 mg/kg)	6	10 (1.5)	12 (1.9)	37 (9.7)	9 (2.3)		0.59 (0.06)
9	2-PMPA (0.2 mg/kg)	9	14 (1.3)	14 (1.9)	19 (2.0)	13 (1.9)		0.19 (0.09)
10	2-PMPA (10 mg/kg)	6	19 (3.2)	18 (3.7)	19 (3.4)	9 (0.8)		0.32 (0.05)
11	2-PMPA (50 mg/kg)	10	12 (1.0)	13 (1.0)	21 (2.1)	11 (1.4)		0.34 (0.03)
12	2-PMPA (100 mg/kg)	9	13 (1.9)	12 (1.5)	31 (3.0)	10 (1.3)		0.51 (0.05)
13	2-PMPA (100 mg/kg)+ LY95 (2 mg/kg)	10	12 (1.7)	13 (2.0)	27 (2.3)	18 (2.4)		0.21 (0.04)
14	2-PMPA (100 mg/kg) [POST AS]	6	16 (1.6)	14 (1.5)	16 (4.2)	9 (1.8)		0.23 (0.07)
15	HET/saline	7	15 (2.9)	15 (3.4)	16 (3.1)	13 (2.5)		0.07 (0.05)
16	GCPII KO/saline	6	14 (3.4)	15 (3.4)	19 (3.8)	7 (2.1)		0.44 (0.08)
17	HET/2-PMPA(100 mg/kg)	7	12 (1.5)	13 (1.9)	31 (7.3)	14 (2.6)		0.30 (0.08)
18	GCPII KO/2-PMPA(100 mg/kg)	6	13 (1.9)	14 (2.1)	18 (3.0)	8 (2.7)		0.41 (0.07)

FO(1) = familiar object 1; FO(2) = familiar object 2 (these objects are identical in shape, size and color); NO = novel object which differs in shape and color from the familiar objects. LY95 = LY341495.