

# Effects of 4-aminopyridine on classical conditioning of the rabbit (*Oryctolagus cuniculus*) nictitating membrane response

Desheng Wang, Deya S. Darwish and Bernard G. Schreurs

A large body of data suggests that potassium channels may play an important role in learning and memory. Previous in-vitro research in a number of species including *Hermisenda* and the rabbit suggests that a 4-aminopyridine-sensitive transient potassium channel may be involved in classical conditioning. We investigated the effects of in-vivo 4-aminopyridine administration (0.5 mg/kg) on classical conditioning of the rabbit nictitating membrane response using a battery of tests designed to assess the associative, sensory, and motor contributors of 4-aminopyridine to responding. 4-Aminopyridine enhanced both classical conditioning and conditioning-specific reflex modification compared with a saline vehicle control, and these effects had several nonassociative components including an increase in the frequency of responding to both the conditioned and the unconditioned stimuli, suggesting a sensitizing effect of the drug. Although 4-aminopyridine can have peripheral effects, it may also modify cerebellar excitability or

hippocampal neurotransmitter balance resulting in heightened responsiveness to stimulation. *Behavioural Pharmacology* 17:319–329 © 2006 Lippincott Williams & Wilkins.

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## Introduction

A growing body of experimental data suggests that transient potassium channels may be involved in learning and memory by mediating several physiological functions including: regulation of membrane excitability (Alkon, 1983; Farley and Alkon, 1985; Disterhoft *et al.*, 1986; Schreurs *et al.*, 1998; Wang and Schreurs, 2006), control of neuronal firing patterns (Flores-Hernandez *et al.*, 1994; Andreasen and Lambert, 1995; Hess and El Manira, 2001), repolarization of action potentials, modulation of short-term synaptic excitation (Poolos and Johnston, 1999; Johnston *et al.*, 2000), induction of synaptic plasticity (Schreurs *et al.*, 1997; Ramakers and Storm, 2002), regulation of calcium channels (Etzion and Grossman, 1998, 1999), and release of neurotransmitters (Schweizer *et al.*, 2003; Cassel *et al.*, 2005; Gasque *et al.*, 2005). Our previous research (Schreurs *et al.*, 1998), as well as work by others (Alkon, 1983; Cowan and Siegel, 1984; Acosta-Urquidi and Crow, 1993), indicates that the inactivation of transient potassium channels may be involved in classical conditioning. We reasoned that if we could block these transient potassium channels with 4-aminopyridine (4-AP) in a manner analogous to the way these channels are thought to be inactivated during normal learning, we might be able to enhance classical conditioning of the rabbit nictitating membrane response (NMR).

Pharmacological studies in animals show that the transient potassium channel blocker 4-AP and its derivatives (e.g. 3,4-diaminopyridine) may improve cognitive deficits when memory is impaired by scopolamine (Poorheidari *et al.*, 1998), electroconvulsive shock (Inan *et al.*, 2000), minoxidil (Banchelli *et al.*, 2000), aluminum toxicity (Yokel *et al.*, 1994), hypoxia (DeNoble *et al.*, 1990), or aging (Barnes *et al.*, 1989). In the only rabbit classical conditioning study to use 4-AP, Yokel *et al.* (1994) showed that 4-AP (0.1 mg/kg) facilitated conditioning in rabbits with aluminum-induced toxicity. No examination of the effects of 4-AP on classical conditioning in normal adult rabbits has been carried out, however, nor have potential nonassociative effects of the drug been assessed.

A large body of classical conditioning research investigating the effects of psychoactive compounds on learning and memory conducted by Gormezano and Harvey and their colleagues have implicated drug-mediated changes in sensory processing as the primary mechanism for facilitating or retarding learning and memory (Gormezano and Harvey, 1980; Harvey and Gormezano, 1981; Schindler *et al.*, 1984). For example, Gormezano and Harvey (1980) found that the facilitative effects of lysergic acid diethylamide on classical conditioning

resulted from a drug-induced decrease in tone threshold. Harvey and Gormezano (1981) found that the deleterious effect of haloperidol resulted from a drug-induced increase in tone threshold. In neither study was nonassociative responding to the conditioned stimulus (CS) or responding to the unconditioned stimulus (US) affected by the drug. Interestingly, experiments assessing the modulatory effects of specific serotonin receptor agonists and antagonists on classical conditioning conducted by Romano and Harvey and their colleagues indicate that the effects of these compounds are mediated, at least in part, by drug-induced changes in the amplitude of the unconditioned response (UR) (Romano and Harvey, 1994; Welsh *et al.*, 1998).

In order to determine the effects of 4-AP on the classical conditioning of the NMR in normal rabbits, a number of behavioral tests were used to assess the effects of 4-AP on learning, memory, and sensory processing (Gormezano and Harvey, 1980; Schindler *et al.*, 1984). The tests included a measure of sensitivity and responsiveness to air puff, a difficult learning task or unpaired stimulus presentations, transfer to an easy learning task, and a test of sensitivity to the tone.

## Methods

### Subjects

Twenty-four experimentally naïve, male New Zealand white rabbits (*Oryctolagus cuniculus*) weighing approximately 2.0 kg were housed individually, with free access to food and water, and were maintained on a 12-h light-dark cycle. All procedures followed the guidelines of the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee.

### Apparatus

The apparatus and recording procedures for the rabbit NMR, first described by Gormezano (Gormezano *et al.*, 1962), have been detailed previously (Schreurs and Alkon, 1990; Schreurs *et al.*, 2000). Each rabbit was restrained in a Plexiglas box and trained in a sound-attenuating, ventilated chamber. A stimulus panel containing a speaker and a 10-W house light was mounted at a 45° angle 15 cm anterior to and 15 cm above the subject's head. Ambient noise (65 dB) was provided by an exhaust fan. Air puffs to the cornea of the right eye were delivered by means of a programmable pressure regulator (ER3000; Tescom, Elk River, Minnesota, USA) connected to a 1-mm diameter tube positioned 10 mm from the center of the cornea. Transducing nictitating membrane movements involved a hook and an L-shaped lever attached to a 6–0 nylon loop sutured into but not through the nictitating membrane. The other end of the lever was attached to a rotary encoder connected to a 12-bit A/D converter (5-ms sampling rate; 0.05-mm resolution). Individual nictitating membrane A/D outputs were

stored on a trial-by-trial basis for subsequent analysis. Data collection, analysis, and stimulus delivery were accomplished using a LabVIEW software system (Austin, Texas, USA) (Schreurs *et al.*, 2000).

### Procedure

After being housed for 1 week, 24 rabbits were randomly allocated to four groups ( $n = 6$ ) that comprised the cells of a  $2 \times 2$  factorial design with the factors of drug (4-AP, saline) and pairings (paired, unpaired). Rabbits received 1 day of adaptation, one 60-trial session of air puff testing to assess sensitivity to air puff (pretest), 10 daily sessions of either trace conditioning or unpaired stimulus presentations, followed by another 60-trial session of air puff testing (posttest). The posttest was designed to assess conditioning-specific reflex modification – a change in the reflex that reflects the strength of conditioning and intensity of the US (Schreurs *et al.*, 1995; Schreurs, 2003). Two days after the posttest, all rabbits were given four daily sessions of delay classical conditioning followed by two daily sessions of tone intensity testing to assess auditory sensitivity.

On the adaptation day, the rabbits were prepared for air puff and recording of nictitating membrane movement and then adapted to the training chambers for the length of time of subsequent training sessions (60 min). The US pretest and posttest sessions involved the presentation of four blocks of 15 combinations of five air puff intensities (0.5, 1, 2, 4, or 8 psi) and three durations (25, 50, or 100 ms). The four separately randomized blocks of the 15 stimulus combinations were presented with the restriction that the same intensity or duration could not occur on more than three consecutive trials.

Each of the 10 paired trace conditioning sessions consisted of 60 presentations of a 100-ms, 1-kHz, 82-dB tone CS that was followed by a 500-ms trace interval and then a 100-ms, 4-psi air puff (i.e. 600-ms interstimulus interval). Paired stimulus presentations were delivered, on average, every 60 s (50–70 s range). Each of the 10 unpaired sessions consisted of 60 CS-alone and 60 US-alone presentations that occurred in an explicitly unpaired manner delivered, on average, every 30 s (20–40 s range). Each of the four delay conditioning sessions consisted of 60 presentations of a 400-ms, 1-kHz, 82-dB tone that coterminated with a 100-ms, 4-psi air puff (i.e. 300-ms interstimulus interval).

The two tone intensity testing sessions consisted of the presentation of one of eight 400-ms tone intensities (55, 60, 65, 70, 75, 80, 85, and 90 dB) or a zero intensity (0 dB) that coterminated with a 100-ms air puff. Each tone intensity–air puff pairing was presented eight times as a randomized sequence with each trial delivered, on average, every 60 s (50–70 s range).

A conditioned response (CR) was defined as any extension of the nictitating membrane exceeding 0.5 mm that was initiated after CS onset but before US onset. A UR was defined as any extension of the nictitating membrane exceeding 0.5 mm that was initiated within 300 ms of US onset. Amplitude of a response was scored in millimeters as the maximum extension of the nictitating membrane. Onset latency of a response was identified as the latency in milliseconds from stimulus onset at which a response rose 0.1 mm above the baseline. Peak latency of a response was determined as the latency in milliseconds from stimulus onset to maximum extension of the nictitating membrane. Area of a response was calculated as the total area under the response curve from US onset to the end of the trial.

To overcome the statistical limitations of empty data cells produced by subthreshold air puff intensities without introducing the potential biases of data imputation, we adopted two additional dependent variable measures – magnitude of the response and magnitude of response area – which measure all amplitudes and areas above the baseline regardless of whether a response meets the 0.5-mm criterion (Garcia *et al.*, 2003).

Response topographies for each of the US intensities were averaged across subjects and US durations and examined for differences by comparing the shape of the averaged response (Seager *et al.*, 2003). To provide a statistical measure of the shape of an averaged response, we analyzed for symmetry (skew) and tail size (kurtosis). A significant positive skew value indicates the response has a peak towards the beginning of the response, and a significant negative value indicates the response has a peak more towards the end. A significant positive kurtosis value indicates the response has a long tail, and a significant negative value indicates the response has a short tail. A skew coefficient is considered significant if the absolute value of skew divided by the standard error of skew is greater than 2, and a kurtosis coefficient is considered significant if the absolute value of kurtosis divided by the standard error of kurtosis is greater than 2 (Systat 8.0; SSI, Point Richmond, California, USA). Our previous research indicated that URs on pretest reached their peak just after US onset with a long tail to the right. Such response topographies would yield high, significant, positive values for both skew and kurtosis. After CS–US pairings, however, URs tended to have peaks that were larger and shifted to the right, yielding lower and even significant negative values for both skew and kurtosis (Seager *et al.*, 2003).

### Drugs

4-AP was purchased from Sigma-Aldrich Chemical Co. (St Louis, Missouri, USA). 4-AP (0.5 mg/kg) dissolved in 0.9% sterile saline solution or the saline vehicle was

injected into the marginal vein of the rabbit ear at a volume of 1.0 ml/kg 30 min before each session following adaptation. A 4-AP dose of 0.5 mg/kg was selected because pilot experiments comparing a dose of 0.1 mg/kg with a saline vehicle control in rabbits previously given unpaired stimulus presentations ( $n = 6$ ) indicated that although 0.1 mg/kg had a significant effect on the amplitude of the UR, it had no effect on classical conditioning of the rabbit NMR. Higher doses were not used because they are known to cause cardiovascular side effects including increased blood pressure and sinus arrhythmia at doses as low as 1.0 mg/kg, convulsions at doses as low as 2.0 mg/kg, and death at doses as low as 3.0 mg/kg.

### Data analysis

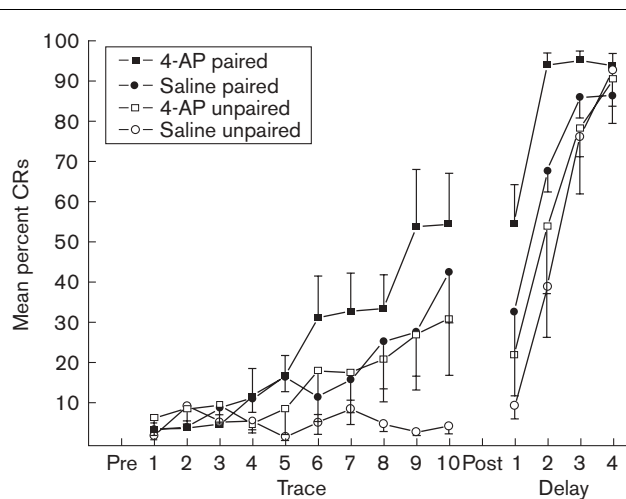
The data were analyzed with repeated-measures analyses of variance using the Systat statistical package, version 8.0.

## Results

### Effects of 4-aminopyridine on the rabbit conditioned nictitating membrane response

Figure 1 depicts mean percentage CRs across the 10 days of trace conditioning and 4 days of delay conditioning. The figure shows that during both trace and delay conditioning, rabbits in the paired groups responded

Fig. 1



Mean percentage conditioned responses (CRs) across the 10 days of trace conditioning and 4 days of delay conditioning for four groups of rabbits injected with 4-aminopyridine (4-AP) (0.5 mg/kg) or saline and receiving either paired or explicitly unpaired stimulus presentations during trace conditioning and paired stimulus presentations during delay conditioning. Trace conditioning consisted of 10 60-trial sessions in which a 100-ms, 82-dB, 1-kHz tone conditioned stimulus (CS) was presented 600 ms before a 100-ms, 4-psi corneal air puff unconditioned stimulus (US). The unpaired groups received the same stimuli but in an explicitly unpaired manner. Delay conditioning consisted of four 60-trial sessions in which a 400-ms, 82-dB, 1-kHz tone CS coterminated with a 100-ms, 4-psi air puff.

more than rabbits in the unpaired groups and that 4-AP produced higher levels of responding than saline. Closer inspection of response levels during trace conditioning shows the slow rate of CR acquisition for the paired groups and the high level of responding in the 4-AP unpaired group – levels comparable to the saline paired group. Analysis of percentage CRs during trace conditioning yielded significant main effects of pairing [ $F(1,20) = 4.94, P < 0.05$ ] and days [ $F(9,180) = 16.28, P < 0.001$ ], and days  $\times$  drug interaction [ $F(9,180) = 3.66, P < 0.001$ ] and days  $\times$  pairing interaction [ $F(9,180) = 5.67, P < 0.001$ ]. The days  $\times$  drug interaction was attributable to a significant difference between 4-AP and saline in the unpaired groups [ $F(9,90) = 3.02, P < 0.01$ ], but not in the paired groups [ $F(9,90) = 1.67, NS$ ].

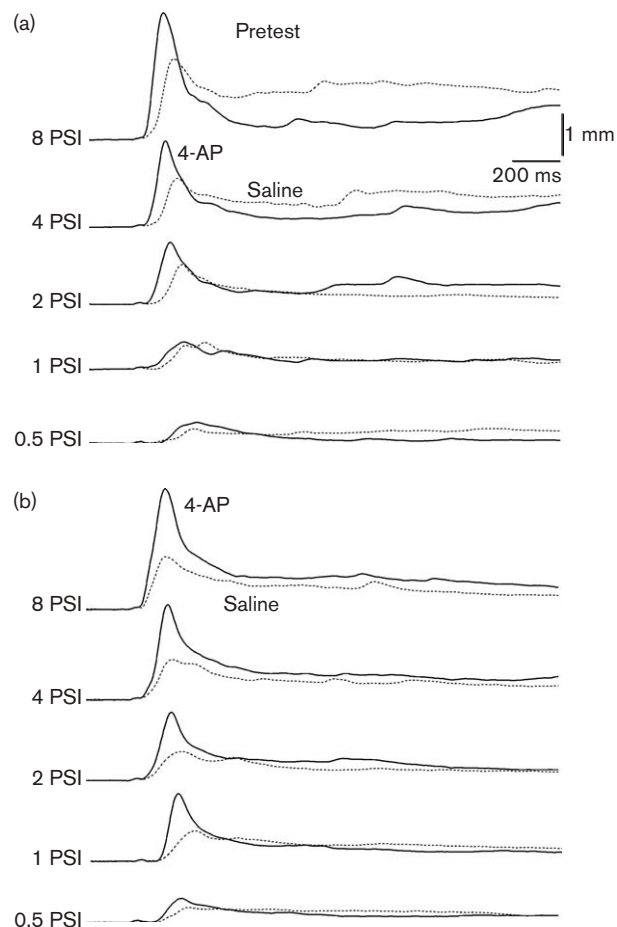
Examination of responding during delay conditioning, when all rabbits received CS–US pairings, shows a continued higher level of responding by rabbits in the 4-AP paired group and the rapid acquisition of CRs by all groups – both paired and unpaired. Analysis of percentage CRs during delay conditioning yielded significant main effects of pairing [ $F(1,20) = 8.43, P < 0.01$ ] and days [ $F(3,60) = 91.198, P < 0.001$ ], and days  $\times$  pairing interaction [ $F(3,60) = 7.83, P < 0.001$ ]. A separate analysis of the paired groups showed a significant main effect of drug [ $F(1,10) = 6.95, P < 0.05$ ] and days [ $F(3,30) = 41.84, P < 0.001$ ], suggesting that rabbits in the 4-AP group responded significantly more than rabbits in the saline group, particularly during the first 2 days of delay conditioning. An analysis of the unpaired groups only yielded a significant effect of days [ $F(3,30) = 53.75, P < 0.001$ ], suggesting that any differences at the end of unpaired stimulus presentations due to nonassociative responding were overcome by the switch to CS–US pairings during delay conditioning.

Taken together, the CR acquisition data indicate that 4-AP produced higher levels of responding than saline and that 4-AP had a clear effect on the level of responding for rabbits in both the paired and unpaired groups, suggesting that the drug may have had nonassociative effects. In order to determine the nature of the potential nonassociative contributors to responding during CR acquisition, we assessed the effects of 4-AP on CS and US responding as well as on reflex modification.

#### Effects of 4-aminopyridine on the rabbit unconditioned nictitating membrane response

The panels of Fig. 2 depict average response topographies to the five US intensities presented during pretest collapsed across the three US durations for rabbits in the 4-AP (solid line) and saline (dotted line) paired (Fig. 2a) and unpaired (Fig. 2b) groups. The figure shows that responses increased in size as a function of US intensity and were typically larger and shifted more to the

**Fig. 2**



Average response topographies to the five unconditioned stimulus (US) intensities presented during pretest collapsed across the three US durations for rabbits in the 4-aminopyridine (4-AP) (solid line) and saline (dotted line) paired (a) and unpaired (b) groups. US intensities are arranged in descending order from 8.0 to 0.5 psi. Pretest (and posttest) sessions involved the presentation of four blocks of 15 combinations of five air puff intensities (0.5, 1, 2, 4, or 8 psi) and three durations (25, 50, or 100 ms). The four separately randomized blocks of the 15 stimulus combinations were presented with the restriction that the same intensity or duration could not occur on more than three consecutive trials.

left in the 4-AP groups than in the saline groups. Analysis of UR frequency yielded significant main effects of US intensity [ $F(4,80) = 41.52, P < 0.001$ ] and drug [ $F(1,20) = 4.80, P < 0.05$ ], suggesting that overall there were more responses elicited by air puff in rabbits injected with 4-AP than those injected with saline. Analysis of UR magnitude only revealed a significant main effect of US intensity [ $F(4,80) = 42.51, P < 0.001$ ], but analysis of magnitude of UR area revealed a significant main effect of US intensity [ $F(4,80) = 17.07, P < 0.001$ ] and a significant intensity  $\times$  drug interaction [ $F(4,80) = 3.06, P < 0.05$ ], suggesting that responses had larger areas in rabbits given 4-AP. Analysis of the

**Table 1** Analyses of skew and kurtosis of unconditioned response topographies

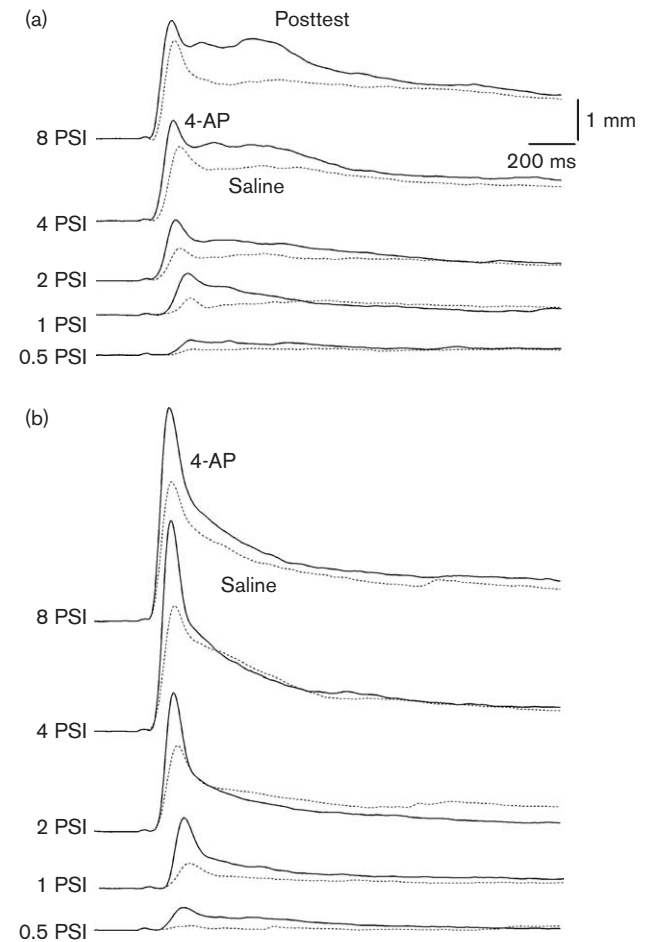
Intensity (psi)	Skew				Kurtosis			
	Pretest		Posttest		Pretest		Posttest	
	4-AP	Saline	4-AP	Saline	4-AP	Saline	4-AP	Saline
Paired								
8	++	--	-	-	++	+		+
4	++	-	-	-	++	+		+
2	++	++		--	++	++	+	+
1	+	++	+	-	+	+	+	+
.5	++	--	-	--	+	+	-	+
Unpaired								
8	++	+	++	+	++	+	++	+
4	++		++	+	++	+	++	+
2	++	+	++	+	++	+	++	+
1	++	-	++	+	++		++	+
.5	++	-	+	+	++		+	-

+ single and ++ double digit significant positive skew or kurtosis; - single and -- double digit significant negative skew or kurtosis. 4-AP, 4-aminopyridine.

skew and kurtosis of the average responses summarized in Table 1 showed consistently high, significantly positive values for the 4-AP groups and variable sometimes negative values for the saline groups on pretest.

The panels of Fig. 3 depict average response topographies to the five US intensities presented during posttest collapsed across the three US durations for rabbits in the 4-AP (solid line) and saline (dotted line) paired (Fig. 3a) and unpaired (Fig. 3b) groups. The figure shows that responses increased in size as a function of US intensity and were typically larger in the 4-AP groups than in the saline groups. The figure also shows that responses in the paired groups were smaller in magnitude but not as narrow as responses in the unpaired groups. Analysis of UR frequency once again yielded a significant main effect of US intensity [ $F(4,80) = 66.80, P < 0.001$ ] and of drug [ $F(1,20) = 8.63, P < 0.01$ ], as well as a significant US intensity  $\times$  drug interaction [ $F(4,80) = 2.79, P < 0.05$ ], suggesting that more responses were elicited by air puff in rabbits injected with 4-AP, regardless of whether the rabbits were in the paired or unpaired group. Analysis of UR magnitude revealed significant main effects of US intensity [ $F(4,80) = 51.28, P < 0.001$ ] and drug [ $F(1,20) = 4.92, P < 0.05$ ], and US intensity  $\times$  group interaction [ $F(4,80) = 3.29, P < 0.05$ ], confirming that responses were larger as a result of 4-AP injections and that responses were larger for rabbits in the unpaired groups. An analysis of magnitude of UR area only revealed a significant main effect of US intensity [ $F(4,80) = 70.77, P < 0.001$ ]. Analysis of the skew and kurtosis (Table 1) of the average response for the paired groups showed values that were quite variable with a considerable number of significant negative values for both the 4-AP and the saline groups. In contrast, analysis of skew and kurtosis for the unpaired groups yielded high, significantly positive

**Fig. 3**

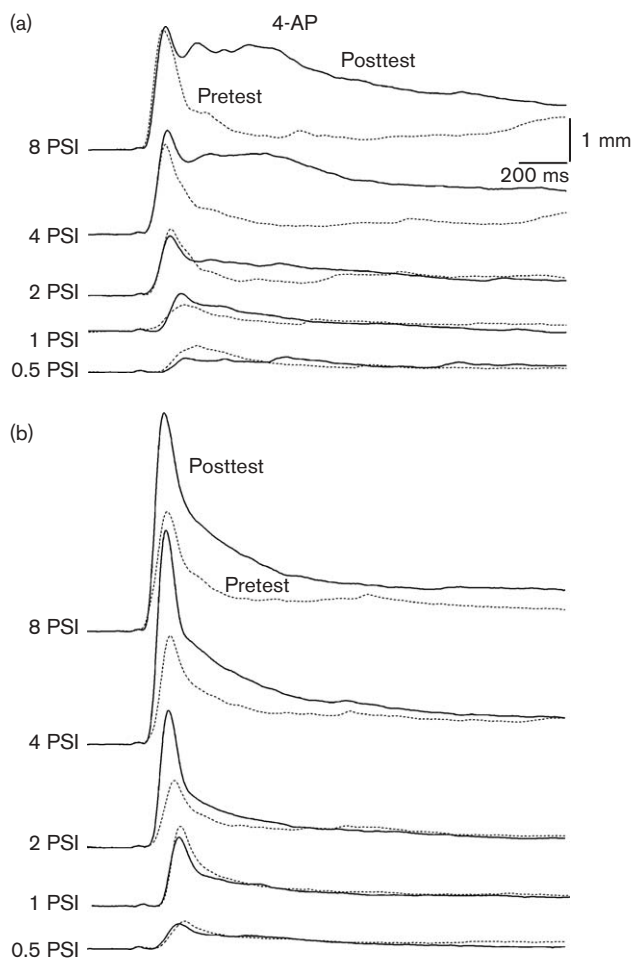


Average response topographies to the five unconditioned stimulus (US) intensities presented during posttest collapsed across the three US durations for rabbits in the 4-aminopyridine (4-AP) (solid line) and saline (dotted line) paired (a) and unpaired (b) groups. US intensities are arranged in descending order from 8.0 to 0.5 psi.

values for the 4-AP group and only slightly lower values for the saline group.

A comparison of the panels of Figs 2 and 3 shows that rabbits had larger responses on posttest than on pretest and, depending on whether or not they had been injected with 4-AP, the nature of those differences varied between rabbits in the paired and unpaired groups. The panels of Figs 4 and 5 are a rearrangement of Figs 2 and 3 and provide direct comparisons of pretest (dotted lines) and posttest (solid lines) for the 4-AP paired (Fig. 4a) and unpaired (Fig. 4b) groups and for the saline paired (Fig. 5a) and unpaired (Fig. 5b) groups. Figure 4 shows clear differences between pretest and posttest for both the 4-AP paired and the unpaired groups, but the nature of those differences depends on whether the rabbits

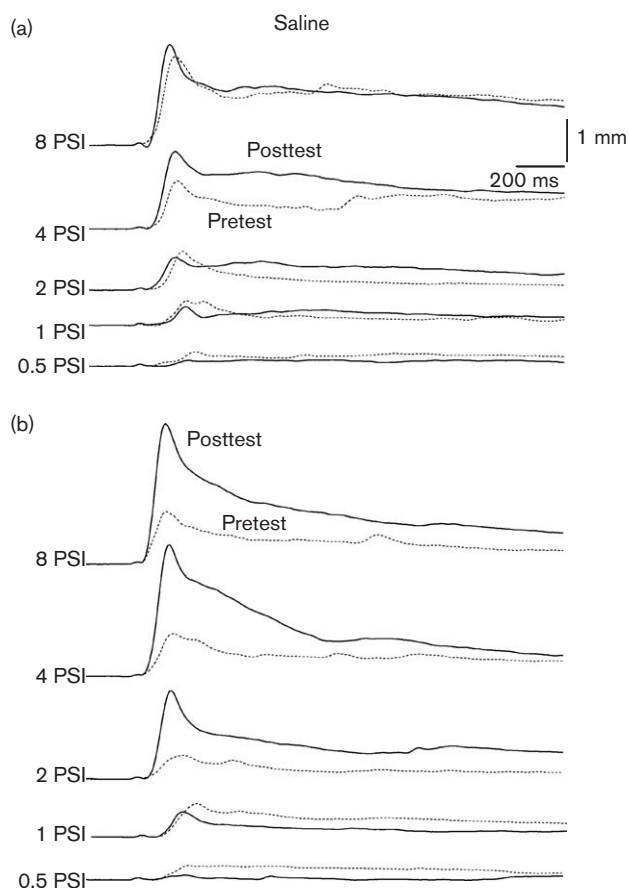
Fig. 4



Average response topographies to the five unconditioned stimulus (US) intensities presented during pretest (dotted line) and posttest (solid line) collapsed across the three US durations for rabbits in the 4-aminopyridine (4-AP) paired (a) and unpaired (b) groups. US intensities are arranged in descending order from 8.0 to 0.5 psi.

received paired or unpaired stimulus presentations. Figure 4a depicts posttest responses for the 4-AP paired groups that were extremely broad with large areas compared with the pretest responses. This is the type of change in response topography from pretest to posttest that we have identified as characteristic of conditioning-specific reflex modification (e.g. Schreurs, 2003). In contrast, Fig. 4b shows posttest responses for the 4-AP unpaired groups that were almost as narrow as pretest responses with larger amplitudes on posttest. Analyses of UR frequency, magnitude, and magnitude of the area for the 4-AP groups revealed significant main effects of US intensity for all three dependent variables [ $F_s(4,80) > 51.00$ ,  $P_s < 0.001$ ]. Only for magnitude of the UR area were there significant interactions of US intensity  $\times$  test [ $F(4,80) = 17.63$ ,  $P < 0.001$ ] and

Fig. 5

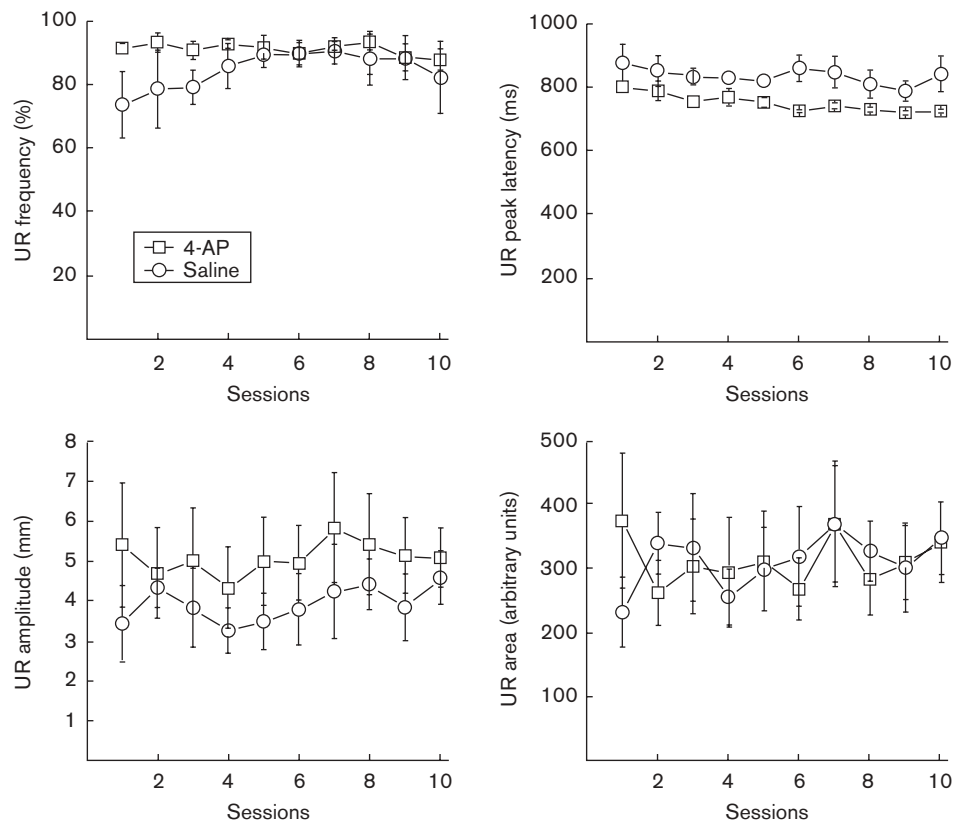


Average response topographies to the five unconditioned stimulus (US) intensities presented during pretest (dotted line) and posttest (solid line) collapsed across the three US durations for rabbits in the saline paired (a) and unpaired (b) groups. US intensities are arranged in descending order from 8.0 to 0.5 psi.

group  $\times$  test [ $F(1,20) = 6.01$ ,  $P < 0.05$ ], confirming that rabbits in the 4-AP paired group had response topographies that were broader and shifted to the right compared with rabbits in the 4-AP unpaired group. This was further confirmed by analyses of skew and kurtosis of the average pretest and posttest responses shown in Table 1 that revealed changes from high significant positive values on pretest to significant negative values on posttest for the paired group but not for the unpaired group.

The panels of Fig. 5 also show clear differences between pretest and posttest for both the saline paired and the unpaired groups and, once again, the nature of the differences depends on whether the rabbits received paired or unpaired stimulus presentations. Figure 5a depicts average pretest and posttest responses for the saline paired group that do not appear to differ greatly. In

Fig. 6



Mean unconditioned response (UR) frequency, peak latency, amplitude, and area for each of the 10 days of unconditioned stimulus (US) presentations for rabbits in the 4-aminopyridine (4-AP) and saline unpaired groups. Each of the 10 unpaired sessions consisted of 60 conditioned stimulus (CS)-alone and 60 US-alone presentations that occurred in an explicitly unpaired manner delivered, on average, every 30 s (20–40 s range).

contrast, Fig. 5b shows average posttest responses for the saline unpaired group that were larger than average responses on pretest. Analyses of UR frequency, magnitude, and magnitude of the area for the saline groups revealed significant main effects of US intensity for all three dependent variables [ $F_s(4,80) > 34.20$ ,  $P_s < 0.001$ ], and significant interactions of US intensity  $\times$  test [ $F_s(4,80) > 5.27$ ,  $P < 0.001$ ] for UR frequency and magnitude of UR area; group  $\times$  test [ $F(1,20) = 6.01$ ,  $P < 0.05$ ] for magnitude of UR area; and US intensity  $\times$  group  $\times$  test [ $F_s(4,80) > 3.01$ ,  $P_s < 0.05$ ] for UR magnitude and magnitude of UR area. These latter interactions confirm that the difference between responding on pretest and posttest for rabbits in the saline unpaired group were larger than the differences for rabbits in the saline paired group.

Taken together, the US testing data indicate that 4-AP facilitated increases in UR frequency, amplitude, and area both on pretest and on posttest. The facilitative effects of 4-AP on pretest suggest that 4-AP sensitized responding to a range of US intensities. In effect, it is as though the

US intensity was perceived to be greater for the rabbits in the 4-AP groups. To determine whether this effect also occurred during conditioning, we examined the frequency, amplitude, peak latency, and area of responding to presentations of the 4-psi US to rabbits in the unpaired groups during trace conditioning.

Figure 6 depicts UR frequency, peak latency, amplitude, and area for each of the 10 days of US presentations for rabbits in the 4-AP and saline unpaired groups. The figure shows that rabbits in the saline unpaired group initially responded at a slightly lower frequency than rabbits in the 4-AP unpaired group. In addition, rabbits in the 4-AP unpaired group had shorter peak latencies than rabbits in the saline unpaired group. No differences seem to exist in the UR amplitude or UR area between the 4-AP and saline unpaired groups. Analyses of the UR-dependent variables revealed significant main effects of drug [ $F(1,10) = 9.54$ ,  $P < 0.05$ ] and days [ $F(9,90) = 2.31$ ,  $P < 0.05$ ] for peak latency, and a significant drug  $\times$  days interaction [ $F(9,90) = 2.61$ ,  $P < 0.05$ ] for UR frequency. The main effect of drug for the peak latency of

responding indicated that UR rise times were shorter for the 4-AP group across all 10 days of unpaired stimulus presentations. Post-hoc contrasts of the drug  $\times$  days interaction for UR frequency, however, failed to isolate the differences in UR frequency to any specific day (largest difference on day 3,  $F = 4.05$ ,  $P < 0.06$ ). In fact, the apparent differences in UR frequency between the two groups were attributable to a single subject in the saline unpaired group that had levels of responding of less than 60% URs over the first 3 days of unpaired stimulus presentations. No significant main effects or interactions of drug or days for UR amplitude or UR area (largest  $F < 1.78$ ) were observed. These UR data suggest that 4-AP produced responses to a 4-psi air puff with consistently faster rise times but interestingly, they were not consistently more frequent nor were they significantly larger.

#### Effects of 4-aminopyridine on tone sensitivity

The significant increase in responding to the CS during the trace conditioning phase by rabbits in the 4-AP unpaired group suggests that 4-AP may have sensitized rabbits to the tone CS. To analyze drug-induced sensitivity to the tone CS, we administered tones of different intensities during 2 additional days of delay conditioning. The panels of Fig. 7 illustrate mean percentage CRs elicited by eight tone intensities in rabbits from the 4-AP and saline paired groups on day 1 and day 2 (top panels) and rabbits from the previously unpaired groups (bottom panels). Inspection of the panels illustrates clearly that the level of responding for all groups increased as a function of tone intensity and that there tended to be more responding to tones of lower intensity by rabbits administered 4-AP in both the paired and the previously unpaired groups on day 2 of tone intensity testing. These observations were corroborated by an analysis of variance that yielded a significant main effect of CS intensity [ $F(8,160) = 251.47$ ,  $P < 0.001$ ], and significant interactions of days  $\times$  CS intensity [ $F(8,160) = 2.32$ ,  $P < 0.05$ ] and drug  $\times$  days  $\times$  CS intensity [ $F(8,160) = 4.99$ ,  $P < 0.001$ ]. Interestingly, there appear to be absolutely no differences in the levels of responding at or above the CS training intensity on either day but differences emerged on day 2 of testing at the lowest CS intensities, suggesting a drug-induced change in threshold. The fact that there was more responding to the lower CS intensities on day 2 also suggests that there may have been some generalization decrement on the first day of tone intensity testing as a result of the transition from the use of a single CS intensity during delay conditioning to multiple CS intensities during CS intensity testing.

#### Discussion

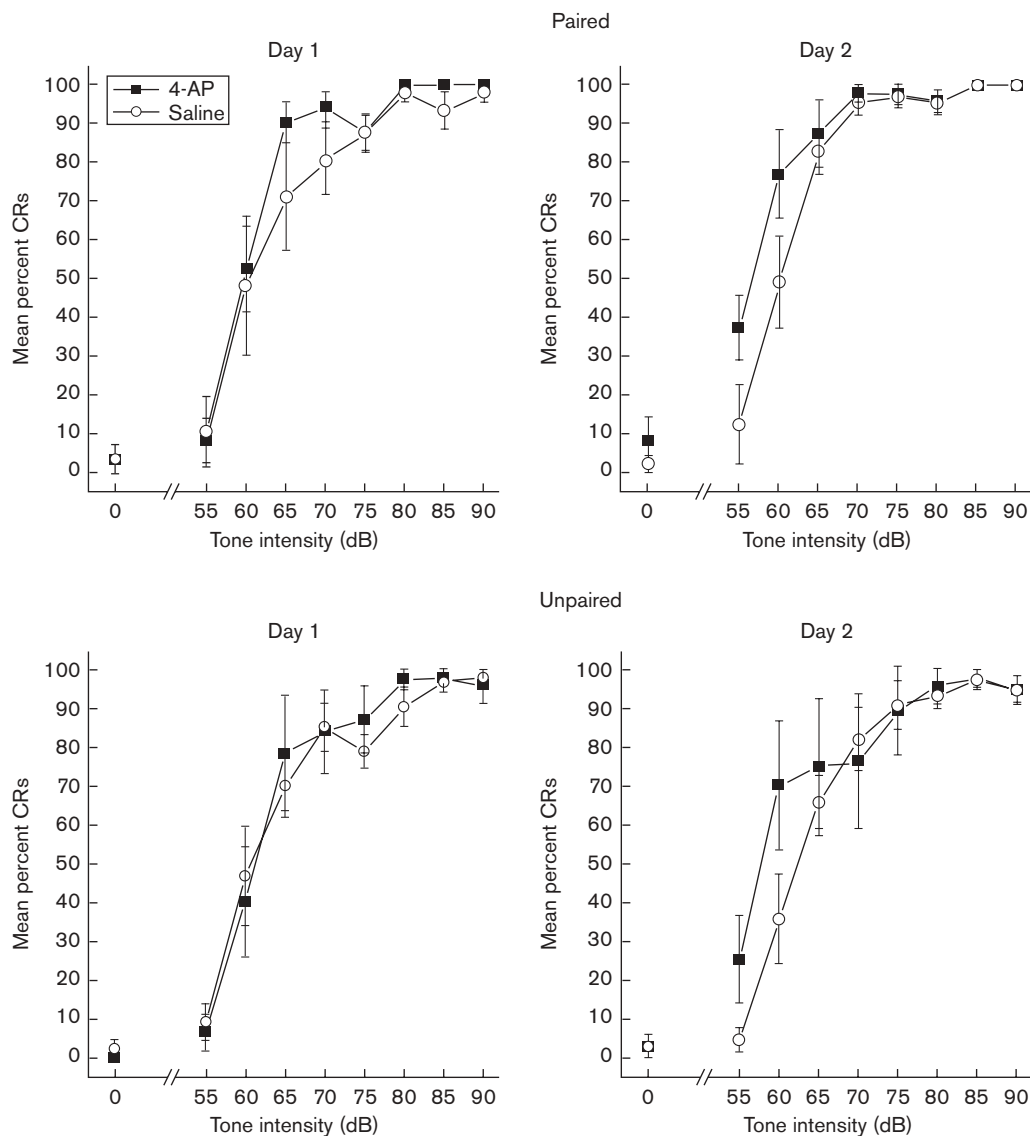
The principal finding of the present study was that 4-AP enhanced both classical conditioning and conditioning-

specific reflex modification as a result, in part, of nonassociative effects of the drug. Using a battery of within-subjects and between-subjects behavioral tests, it was possible to show that 4-AP increased responding to both the CS and the US. During unpaired stimulus presentations, there was a significant increase in the frequency of responding to the CS. Subsequent tone intensity testing indicated that 4-AP produced a decrease in the threshold for responding. This change in threshold, in the absence of increased responding during US presentations, has been interpreted as an associative effect of a drug (e.g. lysergic acid diethylamide; Gormezano and Harvey, 1980). During US-alone testing, there was a significant 4-AP-induced increase in the frequency of responding to the US and a significant increase in the size of the response. In this case, there was also a significant 4-AP-induced decrease in the peak latency of responding to the training intensity, suggesting faster recruitment of the response. Together, these data indicate that unlike previous drug effects on classical conditioning that have all been specific to either associative CS processing or nonassociative US processing effects, 4-AP increased responsiveness to both the CS and the US, suggesting that the drug produced an overall increase in the gain of the system.

Increased responding to the CS reflected in higher levels of responding to CS-alone trials during unpaired stimulus presentations has typically not been reported following drug manipulations. Increases in responding to the CS during unpaired stimulus presentations are generally interpreted as sensitization or pseudo-conditioning (Gormezano and Kehoe, 1975). Sensitization of the rabbit NMR refers to an increase in responding to a stimulus such as the CS as a result of presentations of a stronger stimulus such as the US (Gormezano, 1966; Gormezano *et al.*, 1983). Given that 4-AP increased the frequency and size of responding during US pretest, the increased intensity of the US may have contributed to the sensitization of the CS. Previous comparisons between paired and unpaired stimulus presentations as a function of manipulations of an aversive US intensity have shown no systematic relationship between US intensity and the level of responding to the CS (Gormezano and Fernald, 1971). An increase, however, was observed in the level of nonassociative responding to the CS as a function of US magnitude in the appetitive rabbit jaw movement response (Sheafor and Gormezano, 1972).

Previous conditioning-specific reflex modification experiments indicate that the size of the conditioning-specific reflex modification effect is a function of the strength of conditioning (Schreurs *et al.*, 1995) and the intensity of the US (Seager *et al.*, 2003). The current data did not reveal a significant difference in the strength of conditioning as a result of 4-AP but did reveal a difference

Fig. 7



Mean percentage conditioned responses (CRs) elicited by eight tone intensities (55, 60, 65, 70, 75, 80, 85, 90 dB) or a zero intensity (0 dB), in rabbits from the 4-aminopyridine (4-AP) and saline paired groups on day 1 and day 2 (top panels) and rabbits from the previously unpaired groups (bottom panels). Tone intensity testing consisted of the presentation of one of the eight 400-ms tone intensities or zero intensity that coterminated with the air puff (300-ms interstimulus interval). Each tone intensity–air puff pairing was presented eight times as a randomized sequence with each trial delivered, on average, every 60 s (50–70 s range).

in the level of conditioning-specific reflex modification as a result of the drug. Given that the current pretest data indicated an overall increase in responding as a result of the drug, it could be argued that the 4-AP-mediated conditioning-specific reflex modification resulted from heightened sensitivity to the US.

Although 4-AP derivatives have been proposed as memory enhancers in humans because of their ability to improve cholinergic transmission (Huff, 1996; Huff *et al.*, 1996; Andreani *et al.*, 2000), the use of 4-AP as a cognitive

enhancer in patient populations has met with mixed results. For example, although Wesseling *et al.* (1984) found that 4-AP improved cognitive function in Alzheimer's patients, as did Huff *et al.* (1996), with an indole-substituted analog of 4-AP, Davidson *et al.* (1988) found no discernable effects in Alzheimer's patients and Smits *et al.* (1994) found no cognitive improvement in patients with multiple sclerosis. In contrast, several studies have shown that 4-AP can be used to treat motor neuron diseases by modulating the muscle and motor unit force on failing motor units in dogs (Pinter *et al.*, 1997) and by

improving conduction through demyelinated axons in multiple sclerosis patients (Schwid *et al.*, 1997; Kalla *et al.*, 2004; Strupp *et al.*, 2004). This suggests that the enhanced learning owing to 4-AP might be due to an effect on motor function. The effects of 4-AP on NMR magnitude and area are certainly consistent with the possibility of altered motor function.

Although questions remain about the mechanisms underlying 4-AP's enhancement of responding, it is clear that these mechanisms are complex. Previous studies have demonstrated the role of 4-AP and its derivatives in modulating neuronal excitability by preventing the efflux of potassium from neurons resulting in depolarization (Salin *et al.*, 1996; Schreurs *et al.*, 1998; Marinelli *et al.*, 2000; Martin *et al.*, 2001; Bikson *et al.*, 2002; Schweizer *et al.*, 2003; White *et al.*, 2003; Alshuaib and Mathew, 2004; Bernard *et al.*, 2004; Gasque *et al.*, 2005; Kroner *et al.*, 2005; Vydyanathan *et al.*, 2005) and by increasing the release of neurotransmitters such as acetylcholine (Andreani *et al.*, 2000; Brandsgaard *et al.*, 2000; Schweizer *et al.*, 2003), norepinephrine (Schweizer *et al.*, 2003; Cassel *et al.*, 2005), dopamine (Brandsgaard *et al.*, 2000), and serotonin (Brandsgaard *et al.*, 2000; Schweizer *et al.*, 2003; Cassel *et al.*, 2005; Hardy *et al.*, 2005). Other studies have shown that behavioral effects of 4-AP may be due to the improvement of the function of Purkinje cells by increasing their excitability and modulating their inhibition of deep cerebellar target nuclei (Schreurs *et al.*, 1998; Strupp *et al.*, 2003, 2004; Wang and Schreurs, 2006). 4-AP has also been shown to attenuate the effects of drugs acting at several neurotransmitter systems including the locomotor activating effects of D-amphetamine, cocaine, and scopolamine and the increases in behavioral activity (grooming, rearing, sniffing, and locomotion) produced by morphine as well as decrease presynaptic D2 cataleptic effects (Liu *et al.*, 1994; Rosenzweig-Lipson and Barrett, 1995; Ries *et al.*, 1996; Brandsgaard *et al.*, 2000; Congar *et al.*, 2002; Davison *et al.*, 2004; Witkin *et al.*, 2004; Kroner *et al.*, 2005). Taken together, it appears that 4-AP produces centrally mediated behavioral effects consistent with blocking transient potassium channels, and that these effects interact with or are modulated by neurotransmitter systems involving dopamine, serotonin, norepinephrine, acetylcholine,  $\gamma$ -aminobutyric acid, and opioids – many of which are present in the hippocampus and modulate hippocampal function.

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