

- derived from the procedure used to determine ocular dominance in single-unit recordings, where the responses to optimal stimuli in the two eyes are compared. It is different from the conventional ocular dominance ratio maps (77), which compare the average rather than the optimal responses; such maps can show an ocular dominance pattern even when orientation maps are poor (Fig. 2F) or when one eye is dominant [see Fig. 4C or figure 1 in (14)].
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Inhibitory Cerebello-Olivary Projections and Blocking Effect in Classical Conditioning

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The behavioral phenomenon of blocking indicates that the informational relationship between the conditioned stimulus and the unconditioned stimulus is essential in classical conditioning. The eyeblink conditioning paradigm is used to describe a neural mechanism that mediates blocking. Disrupting inhibition of the inferior olive, a structure that conveys unconditioned stimulus information (airpuff) to the cerebellum prevented blocking in rabbits. Recordings of cerebellar neuronal activity show that the inferior olive input to the cerebellum becomes suppressed as learning occurs. These results suggest that the inferior olive becomes functionally inhibited by the cerebellum during conditioning, and that this negative feedback process might be the neural mechanism mediating blocking.

Current thinking about associative learning has been profoundly influenced by the phenomenon of blocking initially reported by L. J. Kamin in 1968 (1). In a typical blocking experiment, one conditioned stimulus (CS) ("A") is first extensively paired with an unconditioned stimulus (US) (A-US). Then a second CS ("B") under-

goes a compound conditioning with A and the same US (AB-US). Later, when B is tested, virtually no (or very little) conditioning has occurred to B. However, if A was previously not (or weakly) conditioned with the US, then B (as well as A) accrues substantial associative strength during the compound conditioning phase. Thus, conditioning to B during the compound conditioning is inversely proportional to the magnitude of previous conditioning to A. The blocking effect suggests that if a US is already fully predicted by one stimulus, and if the addition of a new stimulus provides no new information about the US, then the

US will not activate or support the learning process responsible for establishing a new CS-US association (2).

Although blocking has been examined extensively at the behavioral level (3) and several neural models (4) have been proposed, one or more underlying neural mechanisms have yet to be identified. Because the essential neural circuitry involved in classical conditioning of eyeblink or nictitating membrane response in the rabbit has been well characterized (5, 6), this paradigm is ideal for examining the blocking phenomenon at the neuronal level (Fig. 1). Typically, eyeblink conditioning occurs when a CS (for example, tones or lights) is paired with a US (for example, airpuffs), which elicits an unconditioned response (UR; a reflexive eyelid closure). Through CS-US association formation, the animal learns to exhibit a conditioned response (CR) to the CS that mimics the UR, precedes the US in onset time, and peaks at about the time of US onset.

Several lines of evidence suggest that the inferior olive provides the "reinforcing" US input to the cerebellum, which supports eyeblink conditioning (7–11). For example, eyeblink conditioning will develop to a tone CS when using inferior olive stimulation as the US (instead of a peripheral US) (8), and lesioning the inferior olive in previously trained animals results in extinction (9, 10) or abolition (11) of CRs with continued CS-US presentations. An interesting property of neurons in the inferior olive is that they show evoked neural activity to the airpuff US (12) and the periorbital stimulation US (13) during the initial stage of CS-US training (before the animal exhibits any CRs), but not when the animals perform CRs during CS-US trials.

Employing a single-unit recording technique, we examined the complex spike responses of cerebellar Purkinje cells (which receive climbing fiber inputs from the inferior olive) (14) over the course of the behavioral training (15, 16). Recordings were centered over Larsell's HVI, because many Purkinje neurons in this region respond to CS and US presentations (5) and because HVI is importantly involved in eyeblink conditioning (6). Of the 54 cells recorded in rabbits, 31 were recorded in lobule HVI, 12 in anterior lobe (HV), 6 in HVIIA (crus I), and 5 in paramedian lobule (Fig. 2). Sixteen of these cells (10 in HVI, 5 in anterior lobe, 1 in HVIIA) exhibited specific evoked complex spike activity in response to US onset during US-only trials. Of these, 5 were recorded early in training (before animals performed any CRs) and also responded to the US with complex spikes on paired trials, whereas 11 were recorded in trained (CR-performing) animals and did not respond to

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the US with complex spikes. In animals presented with strictly unpaired tone and airpuff trials, 20 of 45 recorded Purkinje cells (12 in HVI, 2 in anterior lobe, 2 in HVIIA, and 4 in paramedian lobule) responded to the US with complex spikes. Thus, similar to inferior olive recording studies (12, 13), it appears that as eyeblink conditioning occurs, the inferior olive's ability to convey US information to the cerebellum becomes functionally suppressed.

It is conceivable that the monosynaptic γ -aminobutyric acid (GABA)-containing projections from the cerebellum (that is, the interpositus nucleus) to the inferior olive (17) may serve a negative feedback function, controlling (or gating) inferior olive activity and thus mediating the blocking effect in eyeblink conditioning (Fig. 1). Consistent with this view, intra-olivary infusions of the GABA antagonist picrotoxin in well-trained rabbits (18) allowed Purk-

inje cells ($n = 3$) to respond to the US with a complex spike even though the animals continued to perform CRs, indicating that the CR-induced inhibition of the inferior olive activity is GABA-mediated (Fig. 3).

To test whether the GABA-containing cerebello-olivary projection is involved in blocking, rabbits were implanted with unilateral guide cannulae just above the contralateral inferior olive (Fig. 4). Animals were subjected to Kamin's standard two-stage blocking procedure: phase I, animals received seven daily sessions of tone-airpuff conditioning; phase II, animals underwent five sessions of tone-light-airpuff compound conditioning while either picrotoxin (PTX) or artificial cerebrospinal fluid (ACSF) was

Fig. 1. A highly simplified putative circuit of eyeblink conditioning based on experimental findings and the gross anatomy of the cerebellum and the brainstem. In brief, the pontine nuclei (via mossy fibers) and the inferior olive (via climbing fibers) convey information about the CS and the US, respectively, to the cerebellum, where CS-US association formation is thought to take place (5, 6). The cerebellum (specifically the interpositus nucleus), in turn, sends monosynaptic GABA-containing projections to the inferior olive (17). According to this model, blocking in eyeblink conditioning will occur when a CS (for example, auditory CS_A) acquires sufficient associative strength to activate the cerebellum, which then inhibits the inferior olive (via the GABA-mediated cerebello-olivary pathway) from US activation. Because the input representing the US can no longer reach the cerebellum, it cannot support conditioning to a new CS (for example, visual CS_B). Thus, the inferior olive (US) input functions as an error-correcting algorithm. Accordingly, if the GABA-containing cerebello-olivary projection is negated, for example, by infusing GABA antagonists directly into the inferior olive, then blocking should be prevented. The auditory and visual regions of the pontine nuclei are delineated by dotted lines. The CR pathway projects from the cerebellum to the eyeblink reflex path that mediates the UR (represented by dashed lines). Open arrows denote modifiable CS-cerebellum synapses.

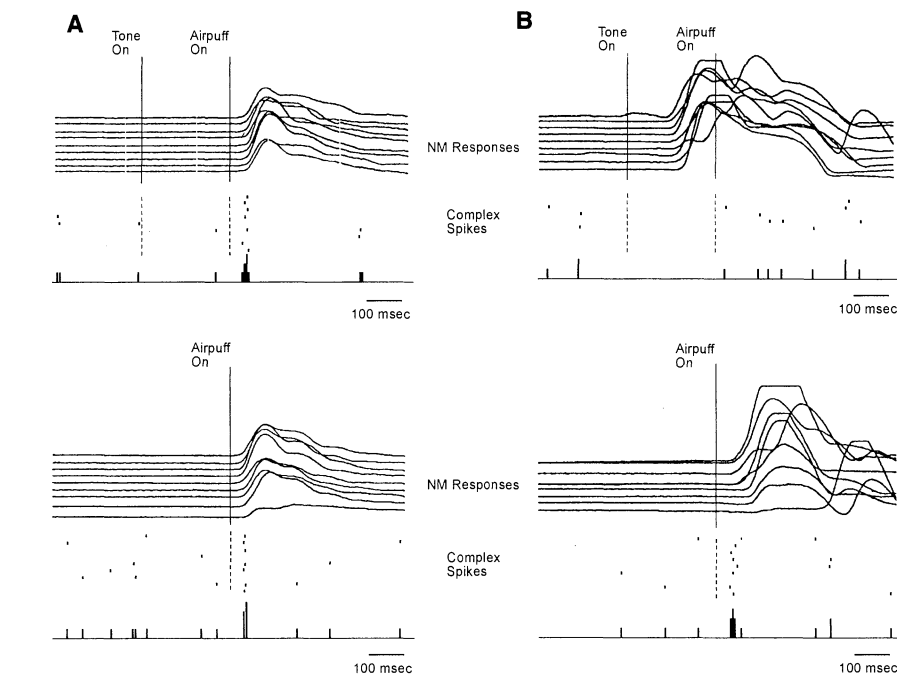
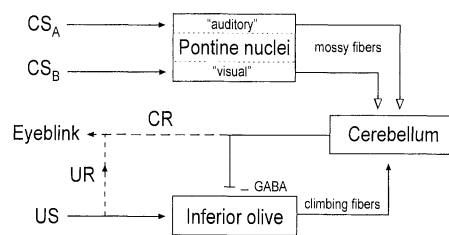


Fig. 2. Examples of a raw complex spike recorded from a Purkinje cell in lobule HVI (lower right), and trial by trial rasters and cumulative peristimulus time histograms (4 ms bins) of discriminated complex spike activity during airpuff-only and tone-airpuff paired trials. (A) In naïve animals, the airpuff-evoked complex spike is observed in both paired (CS-US) and US-only trials. (B) During the CS-US paired trials where the animals display CRs (NM, nictitating membrane), the airpuff-evoked complex spikes are absent [$\chi^2(1) = 8.0$, $P < 0.01$].

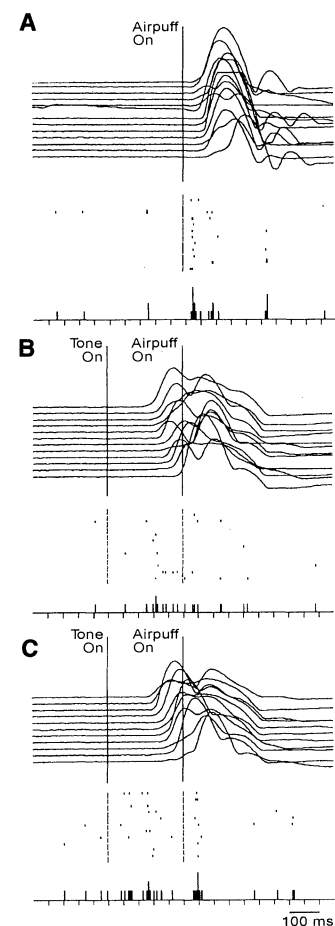


Fig. 3. Example of an isolated Purkinje cell in a well-trained rabbit before and after infusions of PTX into the inferior olive. (A) During the US-only trials, the Purkinje cell responded to the airpuff with a complex spike in a time-locked pattern. (B) The same cell, however, did not respond to the airpuff with a complex spike when the animal displayed CRs during the paired CS-US trials. (C) When PTX was infused into the inferior olive, the cell began to respond to the airpuff with a complex spike even though the animal continued to perform CRs, indicating that the CR-induced inhibition of the complex spikes had been prevented by PTX.

infused directly into the inferior olive (19). Controls experienced only the second phase of the blocking procedure. Afterward, all animals were presented with light-airpuff pairings to assess whether conditioning to the light had accrued during compound conditioning (phase II).

The mean percent CRs during the 7 days

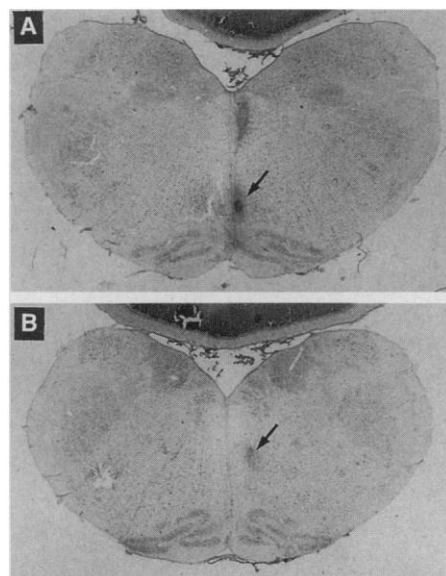


Fig. 4. Transverse brain sections stained with cresyl violet and Prussian blue from rabbits with (A) effective (<1 mm dorsal) and (B) ineffective (~2 mm dorsal) guide cannulae implanted effectively and ineffectively above the dorsal accessory portion of the inferior olive. Arrows indicate cannulae tip positions.

of tone-airpuff conditioning (Fig. 5A), the 5 days of tone-light-airpuff compound conditioning (Fig. 5B), and the 5 days of light-airpuff savings test (Fig. 5C) from PTX, ACSF, and control groups are shown. Both control and PTX animals exhibited significant learning to the light CS compared with the ACSF animals [$F(2,22) = 6.60$, both P 's < 0.01, Newman-Keuls] (Fig. 5C). In fact, the control and PTX groups showed immediate responses to the light CS, and the overall percent CRs to the light CS in the PTX group was not statistically different from controls (56 and 64%, respectively), indicating that blocking did not occur in the PTX group. In contrast, ACSF animals demonstrated blocking; they did not show evidence of conditioning to the light (during compound conditioning) and subsequently learned the CR to the light over 5 days of light-airpuff training. PTX had no effect on the performance of CRs (Fig. 5B) and URs (Fig. 5D) during the compound conditioning, indicating that PTX selectively affected blocking.

It is likely that infusions of PTX into the inferior olive during compound tone-light-airpuff training impeded the tone-induced cerebellar inhibition of US-evoked inferior olive responses, allowing animals to condition to the light CS, thereby preventing blocking. In the controls that did not receive tone-airpuff training beforehand, conditioning to the light stimulus occurred during compound training because there was no cerebellar inhibition of inferior olive activity in re-

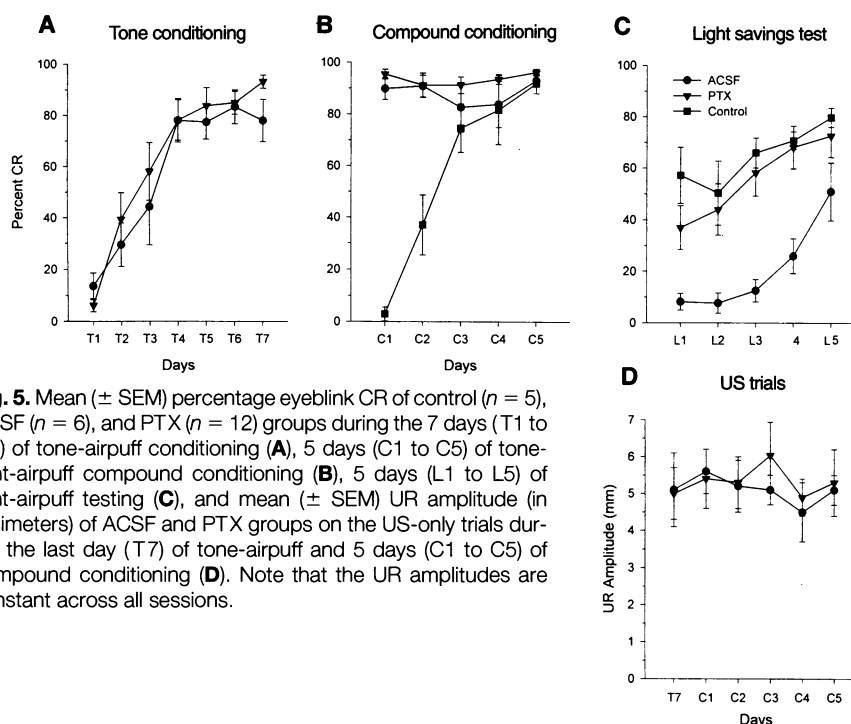
sponse to incoming US information.

Our results indicate that the GABA-containing cerebello-olivary projection (17) plays a crucial role in mediating blocking in eyeblink conditioning. Forms of learning dependent on other structures may employ a similar negative feedback mechanism to regulate the US or "reinforcing" input (20, 21). For example, it has been reported that many dopamine neurons in the substantia nigra and the ventral tegmental area show phasic responses to the delivery of liquid reward in monkeys undergoing a spatial delayed response task. However, once learning is established (that is, the animal learns that a light cue predicts the reward), the delivery of the reward no longer elicits phasic responses in dopamine neurons (21). Such negative feedback circuits in the brain may well provide the neuronal instantiation of behavioral interpretations of blocking (1, 2).

The importance of responding selectively to those stimuli (for example, CSs) which reliably predict biologically significant events (for example, USs) offers a functional explanation for associative learning in an animal's adaptation to its environment. In the interest of efficiency and simplicity, animals should avoid forming associations with other stimuli that provide no new information about the US. The behavioral phenomenon of blocking, which appears to use a heuristic negative feedback process, serves to circumvent such redundant learning.

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 14. Purkinje cells are the sole output neurons of the cerebellar cortex that project to cerebellar deep nuclei (such as the interpositus nucleus) [M. Ito, *The Cerebellum and Neural Control* (Raven Press, New York, 1985)], and these neurons demonstrate synaptic plasticity (for example, long-term depression) hypothesized to be involved in eyeblink conditioning (5).
 15. A circular craniotomy (6 mm diameter) was performed over the left cerebellum, centered over Larsell's HVI (4.5 mm lateral and 1.0 mm rostral to the lambda skull suture). A stainless steel recording base for a hydraulic microdrive (Kopf 650) was cemented over the craniotomy with dental acrylic and stainless steel skull screws. Electrode penetrations could be precisely located anywhere within the 6-mm opening. All surgeries were performed under ketamine HCl (60 mg per kilogram body weight), xylazine (6 mg/kg), and 1 to 2% halothane gas anesthesia. Rabbits ($n = 14$) received 7 days of post-operative recovery.
 16. For recording, a single-unit electrode (Z 64 5 megohm at 1 kHz), was lowered to the surface of the cerebellum and then further lowered in steps of $<5 \mu\text{m}$ to isolate the complex spike activity of a Purkinje cell. The signal was fed into a window discriminator that allowed the number of complex spikes above the noise envelope to be sampled and stored in the computer. Once a unit was isolated, the rabbit was presented with either unpaired tones and airpuffs or paired tone-airpuff trials. Trials were presented until the cell was lost or enough trials were collected to characterize the cell's activity. Small marking lesions were placed at interesting recording sites or at the bottom of electrode tracts to histologically reconstruct the path of electrode penetrations. Single units were recorded from several regions within cerebellar cortex likely to be involved in eyeblink conditioning, including lobule HVI, HV (anterior lobe), HVIIA (crus I), and paramedian lobule. The following criteria were used to identify Purkinje cells: (i) the presence of both simple and complex spikes in the same unit recording; (ii) characteristic activity through different cortical layers (molecular, Purkinje cell, granule cell); and (iii) histological reconstructions—recording sites located within the Purkinje cell layer were identified by Nissl staining. Only the cells that reliably fit each of the above criteria were classified as Purkinje cells.
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 18. For PTX infusions ($1 \mu\text{l}$ of $10 \mu\text{M}$ at a rate of $0.1 \mu\text{l}/\text{min}$), a guide cannulae was implanted into the contralateral dorsal accessory portion of the inferior olive ($n = 2$ animals). The placement of the cannulae was aided by monitoring characteristic inferior olive cell activity (groups of two to three spikes, low spontaneous rates) with a recording electrode protruding from the cannulae. Interestingly, by removing the GABA-mediated inhibition in the inferior olive, PTX seems to increase the overall frequency of complex spike activity recorded in the cerebellum.
 19. Blocking procedures: (Phase I) Two blocking groups (ACSF and PTX) underwent 7 days of tone-airpuff conditioning. Controls were restrained in the training

apparatus for the same amount of time. All groups received intraolivary ACSF infusions on the last day. (Phase II) Animals were then subjected to 5 days of tone-light-airpuff compound conditioning. During this phase, ACSF and PTX ($1 \mu\text{M}$) were infused continuously at a rate of $0.1 \mu\text{l}/\text{min}$ from 15 min before training up to the end of training. (Test phase) Animals were given 5 days of light-airpuff savings testing. Daily training was divided into 10 blocks with each block consisting of 10 trials, totaling 100 trials. With each block, the first and sixth trials were CS-only and US-only trials, respectively, and the remainder were paired trials. The tone CS was 1 kHz, 85 dB, and a duration of 500 ms, the light CS was 45 lux and 500 ms, and the airpuff US was 2.3 N/m^2 and 100 ms. In our preparation, the external eyelids were held open and the corneal airpuff US was delivered

to the temporal region of the cornea, a region not covered by the nictitating membrane at full extension. Both CSs preceded the US by 400 ms and the stimuli coterminated.

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Role of Dynamin in the Formation of Transport Vesicles from the Trans-Golgi Network

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Dynamin guanosine triphosphatases support the scission of clathrin-coated vesicles from the plasmalemma during endocytosis. By fluorescence microscopy of cultured rat hepatocytes, a green fluorescent protein–dynamin II fusion protein localized with clathrin-coated vesicles at the Golgi complex. A cell-free assay was utilized to demonstrate the role of dynamin in vesicle formation at the trans-Golgi. Addition of peptide-specific anti-dynamin antibodies to the assay mixture inhibited both constitutive exocytic and clathrin-coated vesicle formation. Immunodepletion of dynamin proteins also inhibited vesicle formation, and budding efficiency was restored upon readdition of purified dynamin. These data suggest that dynamin participates in the formation of distinct transport vesicles from the trans-Golgi network.

The dynamins comprise a family of 100-kD guanosine triphosphatases that have been implicated in severing clathrin-coated invaginations from the plasma membrane based on the *shibire*^{ts1} mutant of *Drosophila melanogaster* (1) and studies of a mutant dynamin isoform overexpressed in mammalian epithelial cells (2–4). Originally dynamin was thought to be a neuronal specific protein. However, three distinct dynamin genes recently have been identified in mammals: dynamin I (*Dyn1*) is expressed exclusively in neurons (5, 6); dynamin II (*Dyn2*) is found in all tissues (6); and dynamin III (*Dyn3*) is restricted to the testis, the brain, and the lung (7). Each dynamin gene encodes at least four alternatively spliced isoforms (8). Whether all these dynamin gene products function solely at the plasma membrane or also mediate other vesicle scission events at distinct cellular sites is unknown (8). Recently, a dynamin

has been localized to the Golgi complex of mammalian cells by biochemical, immunological, and morphological techniques (9, 10). To provide additional evidence supporting the Golgi localization of a specific dynamin isoform, we linked Dyn2 (spliced form “aa”) to green fluorescent protein (GFP) and expressed it in a rat hepatocyte cell line. Subsequently, its distribution was followed in vivo by fluorescence microscopy (11–13) (Fig. 1). In parallel, untransfected cells were labeled with a Dyn2-specific antibody and a Pan-dynamin antibody (MC63), which recognizes a conserved region of the dynamins (14). A prominent punctate staining at the plasma membrane and the Golgi region was observed with both experimental protocols [GFP-Dyn2 in vivo (Fig. 1, B and D) and endogenous Dyn2 after fixation and immunolocalization (Fig. 1, A, B', C, and D')]. Thus, the transfection process did not alter the distribution of the endogenous Dyn2 compared with untransfected cells. Importantly, the overlap between the two images (Fig. 1) suggests that a Dyn2 isoform is localized to vesicles at both the plasma membrane and the Golgi complex.

To define more precisely the localization of Dyn2 at the Golgi region, cells expressing

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