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Cerebellar Function in Consolidation of a Motor Memory

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Summary

Several forms of motor learning, including classical conditioning of the eyeblink and nictitating membrane response (NMR), are dependent upon the cerebellum, but it is not known how motor memories are stored within the cerebellar circuitry. Localized infusions of the $GABA_A$ agonist muscimol were used to target putative consolidation processes by producing reversible inactivations after NMR conditioning sessions. Posttraining inactivations of eyeblink control regions in cerebellar cortical lobule HVI completely prevented conditioning from developing over four sessions. In contrast, similar inactivations of eyeblink control regions in the cerebellar nuclei allowed conditioning to develop normally. These findings provide evidence that there are critical posttraining memory consolidation processes for eyeblink conditioning mediated by the cerebellar cortex.

Introduction

There is general agreement that the cerebellum is important for motor learning, but the nature of its involvement is far from understood. Fundamental questions remain unanswered as to where and how cerebellar-dependent motor memories are stored. Are they stored within the cerebellar cortex, perhaps as modifications of parallel fiber synaptic efficacies, as first suggested by Marr (1969) and Albus (1971)? Or are they stored in a more distributed form across the cerebellar cortex and at Purkinje cell target neurons in the cerebellar and vestibular nuclei? In vitro analyses have revealed a wide range of plasticities involving different cerebellar and nuclear neurons, but which, if any, of them support motor learning (Hansel et al., 2001)? One system that has been useful for analysis of these questions is classical conditioning of the rabbit eyeblink/nictitating membrane response (NMR) (see Kim and Thompson, 1997; Yeo and Hesslow, 1998). Its dependence upon the cerebellum has been revealed by lesion studies—damage to the cerebellar cortex (Yeo et al., 1985a), cerebellar nuclei (McCormick and Thompson, 1984; Yeo et al., 1985b), or inferior olive (McCormick et al., 1985; Yeo et al., 1986) all abolish or impair NMR conditioning. But these initial lesion studies did not reveal where the motor memory is stored, because they do not dissociate effects upon storage and expression of leaning. Storage and expression can, however, be dissociated by using localized, reversible inactivations of function.

Normal function in the cerebellar nuclei (Krupa et al., 1993), inferior olive (Welsh and Harvey, 1998), and cerebellar cortex (Attwell et al., 2001) is essential for acquisition of NMR conditioning. Inactivating any of these structures during conditioning training results in a failure to acquire conditioned responses (CRs), as measured by performance after recovery from the inactivation. In contrast, inactivation of cerebellar outputs within the superior cerebellar peduncle does not prevent acquisition (Krupa and Thompson, 1995).

Although this set of findings confirms that information storage essential for NMR conditioning is within the cerebellum, its localization at any particular level within the cerebellar circuitry is not disclosed. With the discovery of an inhibitory feedback projection from the cerebellar nuclei to the inferior olive (Andersson et al., 1988; Andersson and Hesslow, 1987a, 1987b), it became clear that the cerebellum has multiple compartments, each containing an olivo-cortico-nucleo-olivary loop. Interference by reversible inactivation at any level in this loop will have consequences for information processing at the other levels (Attwell et al., 2001; Ramnani and Yeo, 1996; Yeo et al., 1997) (see Figure 1). For example, inactivation of olivary output produces marked elevations of Purkinje cell simple spike discharge rates (Benedetti et al., 1983; Colin et al., 1980; Montarolo et al., 1982) that tonically inhibit the cerebellar nuclei and sharply affect the processing of mossy fiber information entering the loop at the cortical level. Similarly, where inactivation of the cerebellar nuclei prevented acquisition and extinction learning (Krupa et al., 1993; Hardiman et al., 1996; Ramnani and Yeo, 1996), we are unable to determine whether these losses relate directly to disturbed function in the cerebellar nuclei or indirectly to disturbed function in the inferior olive or cerebellar cortex. The results of reversible inactivation studies are consistent with views of essential plasticity for eyeblink/NMR conditioning in the cerebellar nuclei, cerebellar cortex, or distributed between these two levels.

An analytic method that overcomes this problem of distributed disturbances of encoding during acquisition training is to target posttraining processes. A consistent feature of memory formation is its transition from a short-lasting, labile form to a longer lasting, stable form through the process of consolidation (McGaugh, 1966; Tully et al., 1994). Before consolidation is complete, memories are still sensitive to trauma and interference, but after consolidation, they become resistant. Clinical examples of posttraumatic amnesia following concussions or other brain insults indicate labile phase durations of minutes to hours, and recent studies have revealed that motor learning also depends upon a consolidation period. Two conflicting motor skills can be learned and retained in the long term only if consolidation for the first task is permitted by delaying training on the second task by 4–5 hr (Shadmehr and Brashers-Krug, 1997). NMR conditioning has a consolidation

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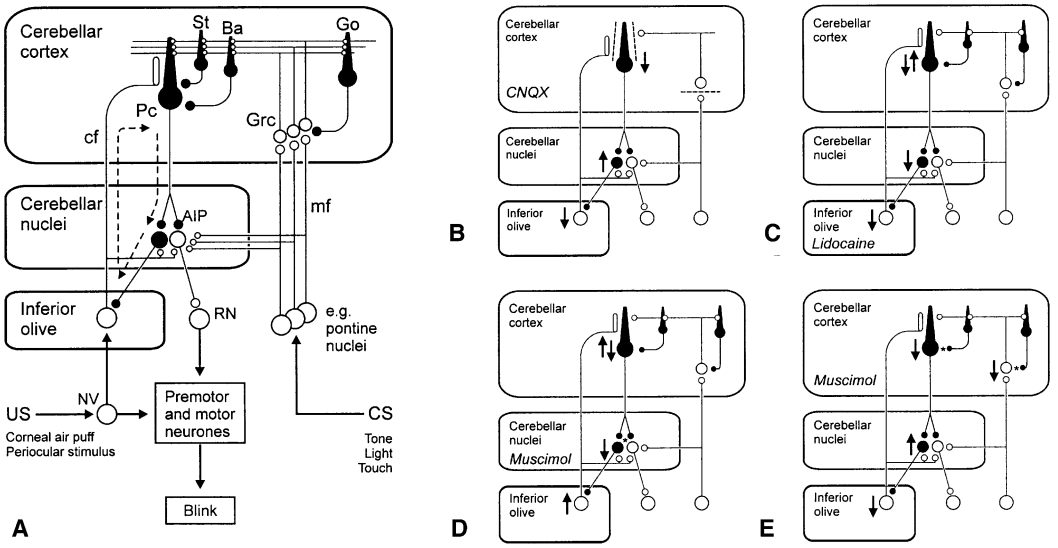

Figure 1. Olivo-Cortico-Nuclear Loops: Inactivation Effects

(A) A model of the cerebellum as a mediator of eyeblink conditioning. CS- and US-related information converges within the cerebellar cortex and within the cerebellar nuclei through mossy fiber and climbing fiber inputs, respectively (for a review, see Yeo and Hesslow, 1998). The olivo-cortico-nuclear loop (OCN) is indicated by dashed arrows. Conventions: excitatory neurons and synapses are shown in white; inhibitory neurons and synapses in black. Abbreviations: AIP, anterior interpositus nucleus; Ba, basket cell; cf, climbing fiber; DAO, dorsal accessory olive; Go, Golgi cell; Grc, granule cell; HVI, cortical lobule HVI; mf, mossy fibers; NV, trigeminal nucleus; Pc, Purkinje cell; pf, parallel fibers; RN, red nucleus; St, Stellate cell.

(B–E) Simplified views of the circuitry shown in (A), with cortical interneurons, multiple mossy fiber inputs, and some brainstem circuits omitted for clarity. Each panel shows how information transmission and excitabilities within the OCN may change after four different interventions. Excitability increases (↑) and decreases (↓) are indicated. Interventions (B), (C), and (D) all prevent acquisition of NMR conditioning. Are acquisition processes disrupted directly in the targeted structure or indirectly through disturbance of the OCN loop?

(B) CNQX infusions in the cerebellar cortex block ionotropic, non-NMDA receptor mediated transmission and block acquisition of NMR conditioning (Attwell et al., 2001). The main targets (shown as barred synapses) are parallel fiber inputs to Purkinje cells (and cortical interneurons; data not shown), climbing fiber inputs to Purkinje cells, and mossy fiber to granule cell synapses. The block of parallel fiber synapses would reduce simple spike activity in Purkinje cells but may not abolish spontaneous activity. Cerebellar nuclear neurons would be partially disinhibited.

(C) Lidocaine infusions in the inferior olive block olivary output and prevent acquisition of NMR conditioning (Welsh and Harvey, 1998). There is a decrease in complex spike activity but an increase in simple spike activity in Purkinje cells (Benedetti et al., 1983; Colin et al., 1980; Montarolo et al., 1982). This dual excitability change is indicated by ↑↓. The predominant simple spike activity leads to depressed nuclear excitability.

(D) Muscimol infusions in the cerebellar nuclei (active at synapses marked with an asterisk) agonise $GABA_A$ receptors and prevent acquisition and extinction of NMR conditioning (Hardiman et al., 1996; Krupa et al., 1993; Ramnani and Yeo, 1996, 1997). Nucleo-olivary inhibition is depressed, so olivary excitability will be increased. At Purkinje cells, increased climbing fiber activity increases complex spike activity with a corollary reduction in simple spike activity (Andersson and Hesslow, 1987b), indicated by ↑↓. Here, we show that, despite disturbance of the OCN loop, posttraining nuclear muscimol does not impair consolidation of conditioning.

(E) Muscimol infusions in the cerebellar cortex agonise $GABA_A$ receptors (at synapses marked with an asterisk) and strongly depress granule cell and Purkinje cell excitabilities. There is consequent disinhibition of the cerebellar nuclei and a depression of olivary excitability. Here, we show that posttraining cortical muscimol blocks consolidation of NMR conditioning.

phase that has been revealed by a posttraining, systemic administration of a variety of drugs. Scopolomine, amphetamine, and chlorpromazine all impair consolidation if administered immediately after conditioning but are without effect if administered 2 hr later (Scavio et al., 1992). Naloxone (Hernandez and Powell, 1983) and ketamine (Scavio et al., 1992) administered after conditioning mildly facilitate consolidation. These systemic interventions indicate a time window for the labile phase of NMR conditioning of about 2 hr, but they do not localize the critical changes to any brain stucture.

Because consolidation depends upon protein synthesis (Matthies, 1989), discrete infusions of a protein synthesis inhibitor (PSI) such as anisomycin can help localize memory storage. But no techniques have been developed to map the effective distribution of a PSI, so this approach would be less useful in dissociating potential consolidation processes in the cerebellar cortex and nuclei, because of their close proximity. Alternative strategies have been used to investigate amygdala-dependent forms of learning. Posttraining intra-amygdala infusions of lidocaine impair the consolidation of contextual fear conditioning (Vazdarjanova and McGaugh, 1999), and similar infusions of the $GABA_A$ agonist muscimol impair inhibitory avoidance conditioning (Brioni et al., 1989; Izquierdo et al., 1990). Muscimol may interfere with consolidation processes by two mechanisms. First, like lidocaine, it depresses activity at the infusion locus, so it may prevent the modulation of consolidation processes at efferent target structures. Second, muscimol may locally disturb GABAergic modulatory processes essential for consolidation.

Here we have used posttraining inactivation of cerebellar circuitry with muscimol to investigate consolidation mechanisms in NMR conditioning. To target such processes, it must be recognized that there are important differences between the temporal structure of consolidation of single-trial learning, such as fear conditioning, and motor learning where skills develop incrementally over many trials. NMR conditioning requires repeated trials and is most efficient in spaced training designs, with short training sessions of a few trials repeated daily (Kehoe and Gormezano, 1974). Typically, with 50 trial sessions, there are no overt conditioned responses (CRs) during session 1, but they develop during the second or third training sessions (Attwell et al., 2001), consistent with the suggestion that each short training session initiates consolidation processes that continue for some time and that each consolidation step builds upon its predecessor. To obtain a clear impairment of consolidation, we have attempted to disrupt consolidation processes after each of four daily conditioning sessions. We used posttraining infusions of muscimol in the cerebellar cortex or in the cerebellar nuclei in order to inactivate neurons with GABAergic synaptic inputs and confirmed the drug localizations with autoradiography. These posttraining inactivations should allow normal encoding during the training/acquisition process, when activity throughout the olivo-cortico-nuclear loop is undisturbed, but will then target putative consolidation processes.

We found that posttraining cerebellar cortical inactivation completely prevented consolidation of NMR conditioning, but cerebellar nuclear inactivations did not. These findings identify a cerebellar process in the consolidation of eyeblink conditioning and dissociate the effects of cortical and nuclear inactivations on conditioning.

Results

Experimental Design

There were three experimental groups. Two groups had infusion cannula guides directed to lobule HVI of the cerebellar cortex, a region critical for the expression and acquisition of nictitating membrane (NM) CRs (Attwell et al., 1999, 2001). Group HVI-M received muscimol infusions in lobule HVI immediately after conditioning sessions, and Group HVI-S received saline control infusions, also immediately after conditioning. A third experimental group, Group CN, had infusion cannula guides directed to the anterior interpositus nucleus of the cerebellar nuclei and received muscimol infusions at this site immediately after conditioning sessions. Pilot studies had indicated that posttraining infusions of muscimol in the cerebellar nuclei did not impair consolidation, so a separate nuclear saline group was not planned; CN and HVI-S groups would be compared.

There were five experimental phases (see Figure 2), each separated by a 3 day rest period. Phase 1 consisted of four daily conditioning sessions of 50 trials. Each session lasted 25 min and contained 45 trials with paired presentation of the conditional stimulus (CS) and unconditional stimulus (US) and five trials with unpaired presentation of the CS alone. The CS was an auditory tone and the US was periocular electrical stimulation. Each conditioning session in Phase 1 was followed immediately by infusions of muscimol (7 nmol) for Groups HVI-M and CN or saline for Group HVI-S. The muscimol (and saline control) infusions would, therefore, be effective during the posttraining putative consolidation period. During Phase 2, all subjects received four further sessions of training, this time without infusions. If consolidation had been impaired during Phase 1 in either the HVI-M or CN groups, then performance would be at baseline levels at the start of Phase 2, and learning would then progress normally as if from the naive condition. As a control procedure, the HVI-S subjects received cortical muscimol infusions (7 nmol) after each of four more daily conditioning sessions during Phase 3. These subjects would reach asymptotic levels of CRs during Phases 1 and 2, so the effects of repeated posttraining muscimol infusions on existing CRs could be assessed in Phases 3 and 4. HVI-M and CN subjects received no infusions during Phase 3.

All subjects should have reached asymptotic levels of CRs in Phase 4. So, finally, in Phase 5 muscimol was again infused in each subject, but this time *before* a conditioning session. The drug effects upon CR performance during this session, and at time points over the next 24 hr, were monitored to assess whether, during this and earlier phases, the drug had been delivered to the appropriate eyeblink control regions in the cerebellar cortex (Groups HVI-M and HVI-S) or cerebellar nuclei (Group CN).

Infusion Sites, Infusion Efficacies, and Cannulation-Related Damage

In order that they properly tested critical cerebellar regions, the cannulation and infusions in each subject needed to satisfy three criteria:

(1) The infusions needed to be restricted to the cerebellar cortex (for HVI-M and HVI-S Groups) or to the cerebellar nuclei (CN Group). Migration of the drug between cortex and nuclei would not properly dissociate cortical and nuclear effects. This criterion was tested by analysis of the [^{3}H]muscimol quantitative autoradiography. The boundaries of the bound drug distribution were determined, and any level above background in the nontarget region was reason to exclude that subject from its experimental group. This deliberately conservative strategy required no assumptions about the correlation between levels of bound drug and their physiological effects.

(2) The muscimol infusions needed to have been effective in blocking function in the critical cerebellar regions. This criterion was tested in Phase 5. Only if muscimol fully blocked expression of conditioned responses for at least some part of postinfusion period was each subject admitted to its experimental group.

(3) The cannulation should not have produced permanent damage sufficient to invalidate assessment of cerebellar function. To enable this control, the Nissl-stained sections were critically examined, and subjects with extensive, cannulation-related cortical damage in HVI or in the interpositus nucleus region were excluded from further analysis.

Of the 14 cortex-implanted subjects, two were re-

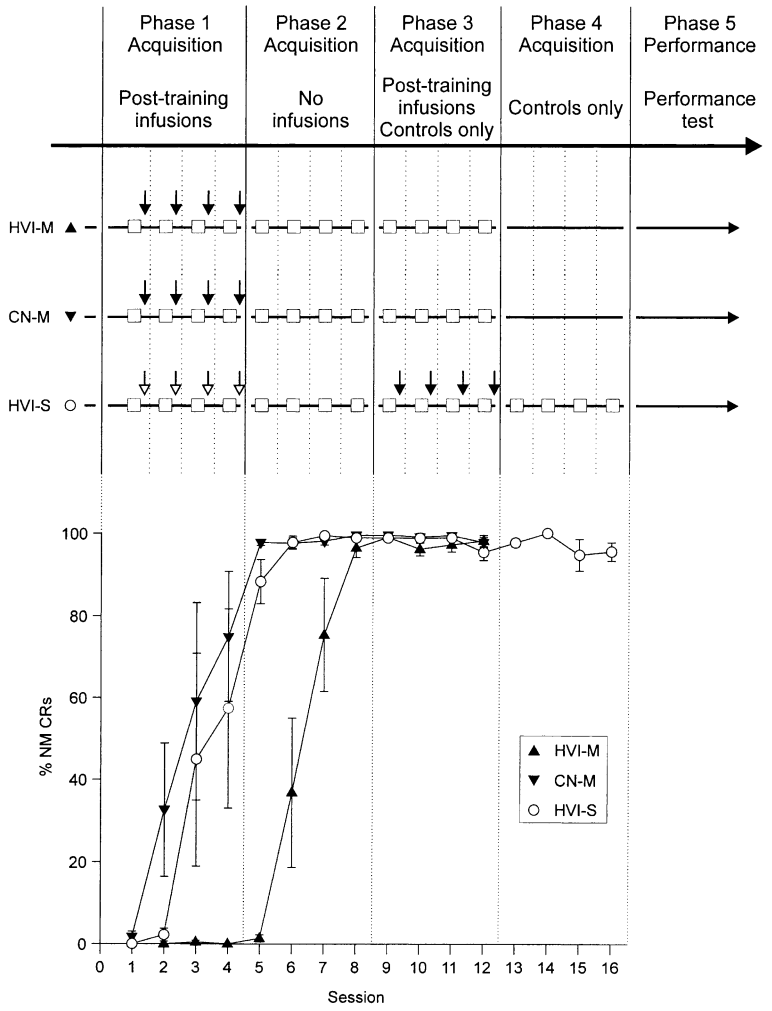

Figure 2. Experimental Design and Effects of Posttraining Cerebellar Muscimol Infusions on Consolidation of NMR Conditioning

Experimental design: each daily session is shown as an open square. Solid vertical lines indicate 3 day rest periods. Posttraining infusions of muscimol (closed arrows) or vehicle (open arrows) are indicated.

Behavioral data: daily, mean session %CRs (± 1 SEM) for the three experimental groups. Cortical control (HVI-S) and nuclear muscimol (CN) subjects developed CRs during Phase 1, but cortical muscimol (HVI-M) subjects did not. HVI-M subjects developed CRs during Phase 2, when muscimol was not given. Posttraining infusions of muscimol given to the HVI-S control subjects during Phase 3, when CR performance was already at asymptote, had no consequences for the maintained expression of CRs during this Phase 3 and in Phase 4.

jected on criterion (2) and three on criterion (3), leaving five HVI-M and four HVI-S subjects. One of the rejected subjects, with incomplete inactivation of HVI, is discussed as subject HVI-X.

Of the nine nucleus-implanted subjects, one was rejected on criterion (1), two on criterion (2), and one on criterion (3), leaving five subjects in the CN group. The subject rejected for drug spread into the cortex is further discussed as subject CN-X.

Posttraining Cerebellar Cortical Muscimol Infusions Prevent Consolidation

Infusions of muscimol (7 nmol in 2 μl) into lobule HVI of the cerebellar cortex immediately following training prevented the development of CRs on successive sessions in Phase 1 (Figure 2, Group HVI-M). During Phase 1 of training, Group HVI-M subjects showed significantly fewer NM CRs than those in the other groups (HVI-S and CN) (Group Comparison 1; Kruskal-Wallis one-way ANOVA, $H = 18.103$, $df = 2$, $p < 0.001$; Dunn's post-hoc test, HVI-S versus HVI-M, $p < 0.05$). Since the drug was not present during the training sessions themselves, these effects cannot be due to impaired encoding during acquisition of the conditioned NMR. Instead, they are a direct action on memory consolidation. This effect was so potent that, throughout Phase 1 of the training, the highest session CR frequencies for the HVI-M subjects was 0.4 ± 0.2 (mean ± 1 SEM) compared with 57.4 ± 12.2 for the control HVI-S subjects, indicating that muscimol infusions can block consolidation almost completely. The infusion effects were fully reversible because all subjects learned the task at normal rates during Phase 2—acquisition for HVI-S control and CN subjects in Phase 1 and for HVI-M subjects in Phase 2 did not differ significantly (Group Comparison 2; Kruskall-Wallis one-way ANOVA, $H = 17.31$, $df = 2$, $p = 0.08$).

It was important to establish whether the failure of the HVI-M subjects to develop CRs was due to true consolidation impairments. One possibility was that normal learning could have been masked by cumulative effects of muscimol treatment, producing long-lasting impairments of performance during Phase 1 and early Phase 2. Alternatively, the enduring effects of muscimol might have induced a compensatory plasticity, perhaps in cortical target neurons in the cerebellar nuclei, to occlude normal learning in Phase 1. Both possibilities were tested by the posttraining cortical muscimol infusions in Group HVI-S subjects during Phase 3. Once learning had reached asymptotic levels, it was no longer sensitive to posttraining cortical muscimol, showing that

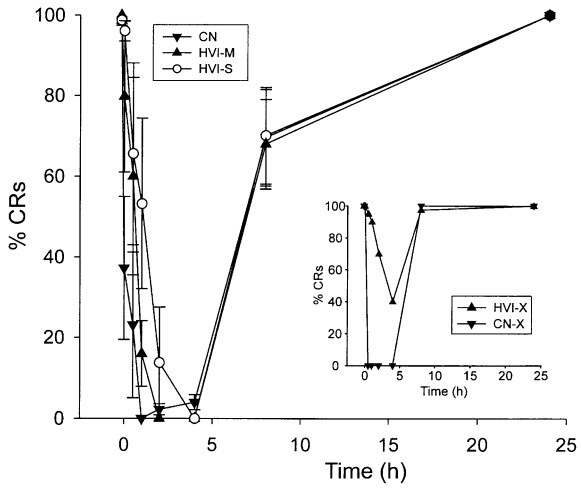

Figure 3. Magnitude and Time Course of CR Depressions after Cortical and Nuclear Muscimol Infusions

Group mean %CRs (± 1 SEM) for 20 trial sessions immediately before, and for time points up to 24 hr after, muscimol infusions given in the Phase 5 performance test. Inset: %CRs for two individual subjects excluded from group analysis. HVI-X had an incomplete block of performance, and consolidation was normal. CN-X had muscimol spread into the cerebellar cortex, and consolidation was impaired.

there could have been no carry-over of the drug from session to session and no cumulative effects that obtained through Phases 3 and 4. Thus, there would have been no carry-over effects and no learning occlusion effects for Group HVI-M during Phases 1 and 2, and the learning impairments of the HVI-M group subjects in Phase 1 are due to disruption of consolidation.

For each subject, the effects of a final cortical muscimol infusion upon performance of CRs were tested in Phase 5. It had been a condition of admission to the experimental groups that these infusions, for the HVI-M and the HVI-S subjects, depressed CR frequency to 0% for at least one block of 10 trials. Figure 3 shows that the rate of onset and duration of drug effects were similar for the HVI-M and HVI-S groups.

In situ autoradiography of [^{3}H]muscimol infusions at the previous dose (see Figure 4) showed that muscimol was confined to the cerebellar cortex in Group HVI-M and that, in all cases, muscimol binding was most prominent in lobule HVI. This location corresponds closely to sites identified as critical for performance and acquisition of NMR conditioning in earlier studies using the non-NMDA ionotropic glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Attwell et al., 1999, 2001).

Posttraining Cerebellar Nuclear Muscimol Infusions Spare Consolidation

Posttraining infusions of muscimol (7 nmol in 1 μl) into the cerebellar nuclei for the four training sessions of Phase 1 did not prevent conditioning. In Group CN subjects, NM CR frequencies developed normally during Phase 1 and did not differ significantly from those of the HVI-S controls (see Group Comparison 1 ANOVA for main effects; Dunn's test NS), though there was a trend toward slightly more rapid acquisition (see Figure 3, Group CN).

There was, therefore, no evidence for muscimol-sensitive consolidation processes within the cerebellar nuclei during the posttraining period. This absence of effect upon consolidation could not have been related to ineffective drug placements. When a similar dose of muscimol was infused in each subject of Group CN during Phase 5 (i.e., when they were fully trained), CR frequency was fully depressed in each subject, and admission to the CN Group had been conditional upon this depression reaching 0% CRs during at least one block of 10 trials. Figure 3 also reveals that the rate of onset and duration of drug effects and the rate of recovery was similar for the CN and HVI-M groups. So the cortical and nuclear muscimol infusions targeted putative consolidation processes over similar temporal windows.

In situ autoradiography of a final infusion of [^{3}H]muscimol revealed bound muscimol in the cerebellar nuclei (see Figure 5), but levels of binding in cortical lobule HVI were at background levels. These localizations confirm that muscimol infusions confined to the cerebellar nuclei do not prevent consolidation of NMR conditioning.

Off-Target Muscimol Infusions Confirm the Importance of Cortical Function in Consolidation

That cortical lobule HVI is a site important for consolidation was further highlighted by two subjects excluded from the group analyses. It was intended that the first of these (subject HVI-X, see Figure 4) was a cortical HVI-cannulated subject. During Phase 1 of the training, it learned at a rate similar to those of control subjects despite receiving posttraining cortical muscimol infusions (97.67% CRs on session 5). However, muscimol infusion failed fully to prevent performance of established CRs during Phase 5 (see inset Figure 3). Autoradiography of radiolabeled drug spread showed that this infusion did not include the rostral parts of lobule HVI, as it did in HVI-M subjects, though it did invade several lobules of the anterior lobe. Results from this subject further confirm that, as for performance and acquisition, rostral lobule HVI is importantly involved in consolidation.

Another case (subject CN-X) was intended as a nuclear-cannulated subject, but its conditioning was severely impaired (0% CRs on session 5). Performance testing in Phase 5 revealed that muscimol had inactivated an area critical for expression of CRs, but autoradiography revealed that the drug was not located in the nuclei but that there was considerable drug binding in lobule HVI (see Figure 5), further supporting the hypothesis that this lobule is important for consolidation.

Discussion

Cerebellar Cortical and Nuclear Inactivations Produce Dissociated Effects upon Consolidation

In previous studies, reversible inactivations of eyeblink/NMR control regions of the cerebellar cortex, cerebellar nuclei, or inferior olive, *before and during* NMR conditioning training have always prevented encoding and acquisition (Attwell et al., 2001; Hardiman et al., 1996;

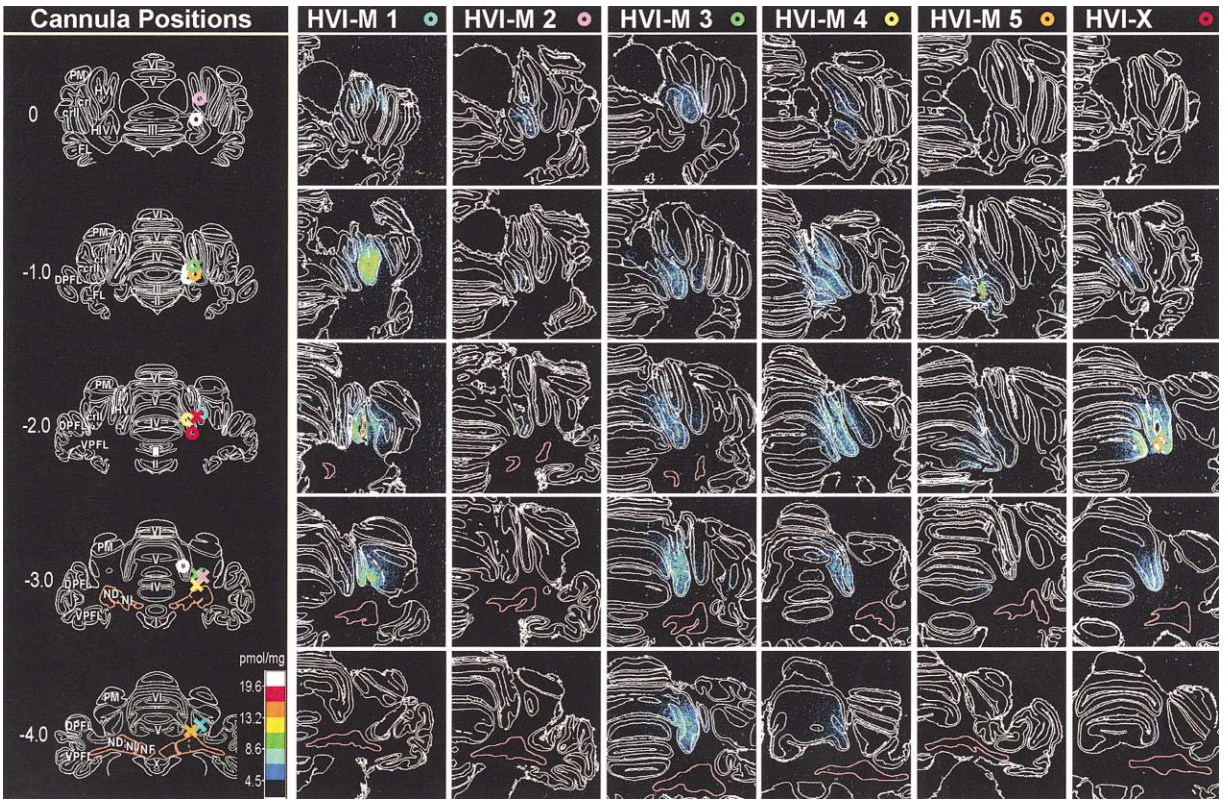

Figure 4. Cannula Tip positions and [^{3}H]Muscimol Distributions following Localized Infusions into Cerebellar Cortex

In column 1, cannula tip locations are shown for all subjects on a series of six standard transverse sections at levels from 0.5 mm anterior to 3.0 mm posterior to skull lambda. White rings indicate locations for the vehicle-infused subjects in the HVI-S control group. Colored rings indicate locations for subjects in the HVI-M group and are identified against each of these subjects in the autoradiography column headings. The cerebellar nuclei are shown with pink boundaries. Columns 2–7 show a series of actual transverse sections for all muscimol-infused subjects at levels corresponding to the standards. Lobule and granule cell boundaries are shown in white. Density of [^{3}H]muscimol binding is color-coded. Densitometry calibration: pmol muscimol/mg tissue equivalent.

Krupa et al., 1993; Welsh and Harvey, 1998). The equivalence of cortical, nuclear, and olivary inactivations in preventing acquisition suggests that information processing throughout the olivo-cortico-nuclear (OCN) loop is disturbed when any nodal point is inactivated. In the present experiment, we now demonstrate a clear dissociation of cortical and nuclear inactivation effects upon motor learning by using inactivations *after* the conditioning training. Inactivations of the cerebellar cortex with muscimol immediately after training substantially prevent consolidation of NMR conditioning in naive subjects, but in contrast, similar posttraining inactivations of eyeblink/NMR control regions of the cerebellar nuclei allow normal learning and consolidation. What are the implications of these findings for our understanding of how this cerebellar-dependent motor memory is stored?

The time course of recovery after muscimol infusions suggests that OCN loop activity was altered for 4–8 hr during the posttraining period. In the HVI-M group, there was inhibition of Purkinje and granule cells, and in the CN group, the cerebellar nuclei were inhibited. Both treatments would have disturbed activity in the OCN loop, but learning was only impaired by the cortical disruption. So the complete loss of learning produced by the intracortical muscimol infusions does not relate to a general disturbance of OCN loop activity. Furthermore, the drug must have impaired consolidation processes specifically, because previously consolidated conditioned responses in the HVI-S subjects were insensitive to posttraining muscimol infusions throughout Phases 3 and 4. The learning is only sensitive to cortical intervention after early acquisition sessions. Are these findings consistent with cerebellar cortical consolidation, cerebellar nuclear consolidation, or both?

On Reversible Inactivations and the Localization of Consolidation

In principle, local infusions of muscimol can disturb consolidation by two mechanisms. First, muscimol may act to disturb consolidation processes postsynaptically at the infusion site by influencing essential local GABA modulation or voltage-gated calcium fluxes. Second, neurons with $GABA_A$ receptors local to the infusion site are deeply inhibited, and information transmission to their efferent targets is blocked or impaired. If consolidation is at these efferent targets and dependent upon input from neurons in the infusion site, then it will be impaired remotely by the inactivation. For our cerebellar cortical and nuclear infusions, we consider both possibilities.

If muscimol affected consolidation directly at the infusion site by disturbing activity in postsynaptic neurons,

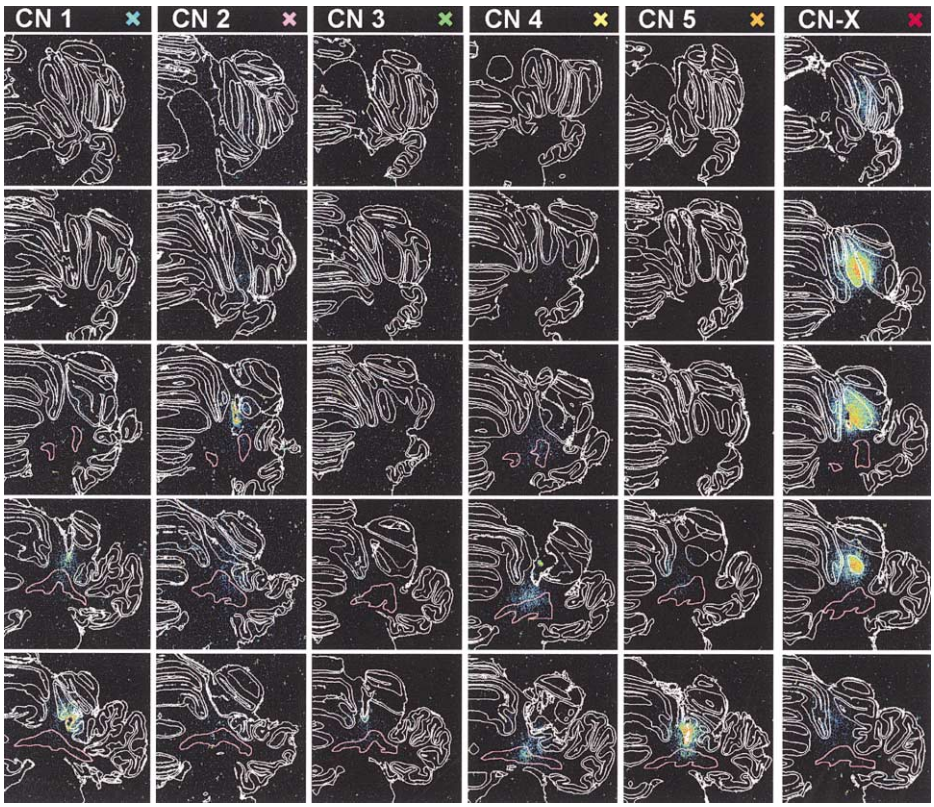

Figure 5. Cannula Tip Positions and [^{3}H]muscimol Distributions following Localized Infusions into Cerebellar Nuclei

Cannula tip positions and muscimol binding in the CN group subjects. Key to cerebellar levels and all other conventions as in Figure 4.

then the interpretation is straightforward. Cortical, rather than cerebellar nuclear, consolidation processes are implicated. However, if muscimol infusions in the cerebellum blocked consolidation by disturbing the transmission of essential information to an efferent target site, where memories are consolidated, then cerebellar cortical muscimol infusions could have prevented consolidation processes either within the cortex, by disturbing intracortical signaling, or in the cerebellar nuclei, by disturbing cortico-nuclear signaling. But this second possibility can be rejected if the effects of nuclear muscimol are considered. Infusions of muscimol in the cerebellar nuclei also disturb cortico-nuclear signaling by potently agonising $GABA_A$ receptors and compromising the Purkinje cell signal to the nuclei, yet consolidation was intact. We conclude that, if muscimol impaired consolidation of NMR conditioning by disturbing the intercellular transmission of specific patterns of information in the posttraining period, then the critical signaling must be intracortical rather than cortico-nuclear, and consolidation of this memory involves an essential cortical process.

It must be recognized, however, that cortical and nuclear muscimol treatments differ in their tonic effects upon the cerebellar nuclei. Cortical muscimol will diminish resting GABAergic modulation of the nuclei to disinhibit their activity whereas nuclear muscimol will increase resting GABAergic modulation of the nuclei to inhibit them (see Figure 1). Current evidence is that disruptions of performance and acquisition of NMR conditioning are indifferent to the direction of GABAergic modulation at the cerebellar nuclei because both are prevented by intranuclear infusions of either the $GABA_A$ agonist muscimol (Krupa et al., 1993; Hardiman et al., 1996) or the $GABA_A$ antagonist picrotoxin (Bao et al., 2002). However, if consolidation processes were uniquely sensitive to the direction of GABAergic modulation change and were impaired by decreases but not increases, then a nuclear consolidation mechanism might be indicated. This possibility can be tested using a $GABA_A$ antagonist.

Adaptation of the vestibuloocular reflex (VOR) is also cerebellar dependent, and it clearly involves plasticity at two levels. There are response changes both at parallel fiber-PC synapses within the flocculus (Ito et al., 1982) and at vestibular afferent synapses on flocculus target neurons (FTNs) in the medial vestibular nucleus (MVN) (Lisberger, 1998). It has been suggested that similar plasticities in cerebellar cortex and at Purkinje cell target neurons in the cerebellar nuclei might also mediate eyeblink/NMR conditioning (Raymond et al., 1996).

Although our findings point toward a cortical plasticity essential for NMR conditioning, they do not rule out plasticity at other levels, including Purkinje cell target neurons in the cerebellar nuclei. First, our inactivations were made at the end of each training session, and they lasted for 4–8 hr. Activity-dependent consolidation processes outside this time window would have been spared, and it is quite possible that nuclear consolidation processes could occur during the training session

itself or, perhaps, several hours after training. Infusions of the PSI anisomycin before training sessions impairs eyeblink conditioning (Bracha et al., 1998), and though it is difficult to be sure whether such infusions are confined to the nuclei and without effect upon acquisition/encoding processes, this finding is consistent with an essential protein synthesis process in the cerebellar nuclei.

Other studies have unmasked short latency, CS-driven responses in previously trained subjects by disabling cerebellar cortical inhibition using cortical lesions or picrotoxin infusions in the cerebellar nuclei (Garcia and Mauk, 1998; Perrett and Mauk, 1995). Although such disinhibition could have unmasked CS-driven excitability changes in other eyeblink control circuits, including those in motor cortex, it is usually assumed that these procedures have unmasked plasticity at the cerebellar nuclei. Recently, nuclear infusions of picrotoxin alone failed to reveal short latency, CS-driven NM responses, but such responses were displayed when nuclear infusions of muscimol were followed by gradually increasing volumes of picrotoxin (Bao et al., 2002). It is suggested that this combination of a competitive $GABA_A$ agonist and a noncompetitive $GABA_A$ antagonist effectively blocked cortical instruction to the nuclei but allowed relatively normal levels of GABA modulation and nuclear excitabilities. These findings have been interpreted as evidence of learning at cortical and nuclear levels: the formation of a basic CS-US association in the cerebellar nuclei and a learned, timing instruction within the cerebellar cortex (Medina and Mauk, 1999, 2000). In these theories, a parallel fiber-Purkinje cell depression is first induced by instruction from the climbing fiber, and then the changed Purkinje cell output instructs a change to mossy fiber collateral inputs to the cerebellar nuclei. Our evidence, from the nuclear muscimol infusion experiment, that compromising Purkinje cell input to cerebellar nuclear neurons during the posttraining period does not prevent consolidation indicates that a previously consolidated cortical change cannot be the consolidation instruction to the cerebellar nuclei within the time window studied. If, as has been proposed, a modified cortical output instructs a nuclear change, then the instruction must occur either within the conditioning session, before cortical consolidation is complete, or several hours later.

Candidate Mechanisms for Cerebellar Consolidation

One well characterized form of cerebellar cortical plasticity is long-term depression (LTD) of parallel fiber synaptic inputs to the Purkinje cells (Ito, 1998). Because LTD can be induced by conjunctive activation of mossy and climbing fiber inputs, it is widely regarded as a candidate plasticity for motor learning where contextual information can be signaled through the mossy/parallel fiber inputs and modified under instruction from the climbing fiber input, as proposed in earlier theories (Marr, 1969; Albus, 1971; Gilbert, 1975; Ito, 1982). The appropriate convergence within lobule HVI of CS- and US-related information through mossy and climbing fiber inputs, respectively (Yeo et al., 1985c; Hesslow et al., 1999), satisfies a simple implementation of such models for NMR conditioning (Yeo and Hesslow, 1998). However, conjunctive LTD is but one of a number of cerebellar plasticities that have been characterized in vitro, and our evidence for cerebellar storage of motor memory does not identify which of them may be critical. If muscimol inactivation operated to prevent consolidation by its direct action on postsynaptic neurons, then it could have disturbed putative consolidation processes at Purkinje cells or at granule cells, because both cell types have $GABA_A$ receptors. If, however, muscimol impeded consolidation by disturbing transmission between cortical neurons in the posttraining period, then other plasticities are implicated. Theta frequency bursting and resonance in granule cells (D'Angelo et al., 2001) could regulate plasticity at mossy fiber to granule cell synapses, and this activity would also have been compromised by muscimol infusions. Very recently, both a long-lasting potentiation (LLP) and a long-lasting depression (LLD) of inputs to Purkinje cells have been demonstrated to control sensory receptive fields. These long-lasting excitability changes have been shown to occur in vivo, and they develop over a period of up to 2 hr after the induction procedure (Jörntell and Ekerot, 2002). Preliminary studies (our unpublished data) indicate that the time window for eyeblink conditioning consolidation processes closely matches that for this delayed LLD/LLP.

Our findings offer evidence for cerebellar mechanisms in consolidation of NMR conditioning in the posttraining period. They point strongly to an intracortical mechanism for consolidation and storage of this motor memory, but a cerebellar nuclear plasticity sensitive to decreased, but not increased, tonic GABAergic modulation is also possible.

Experimental Procedures

Surgery

Male Dutch belted rabbits (2.0–2.2 kg) were implanted with a guide cannula directed toward either the right cerebellar cortical lobule HVI (n = 14) or toward the cerebellar nuclei (n = 9). Following intubation under fentanyl/fluanisone anesthesia (0.1/5.0 mg/kg, i.m.) supplemented with benzodiazepam (0.5 mg/kg, i.v.), each subject received mannitol (10–20 ml, i.v.; 1 ml/min) and enrofloxacin antibiotic (20 mg, i.p.). The head was placed in a stereotaxic instrument with bregma 4.1 mm below lambda, and anesthesia was maintained throughout the operation using halothane (1.5%–2.5%) in a nitrous oxide/oxygen mixture (1:3). The scalp was reflected, and bone and dura was removed to expose the right cerebellar cortex. For HVI implantations, a 26G stainless steel cannula guide was then implanted by visual inspection just below the surface of the lobule. For deep nuclear implants, a 26G cannula guide was placed stereotaxically using stereotaxic coordinates previously determined in pilot studies (5.2 mm posterior to lambda; 4.5 mm lateral to the midline; 11 mm below skull surface). The cannula guide was fixed to the skull with dental cement and the scalp was sutured around the implant. Each animal received analgesic and antibiotic cover for 3 days postoperatively (buprenorphine hydrochoride, 0.1 mg/day; enrofloxacin, 20 mg/day). All subjects were housed individually, allowed food and water ad libitum, and maintained on a 12 hr light/dark cycle for at least 1 week before surgery and throughout the experiment.

Conditioning Protocols

The apparatus and techniques used for conditioning experiments were similar to those first developed by Gormezano et al. (1962) and have been described previously (Yeo and Hardiman, 1992). In each subject, a monofilament loop was sutured in the right nictitating

membrane under local anesthesia (proxymetacaine hydrochloride, 0.5% w/v). Each subject was placed in a Perspex restraining box, and a low-torque potentiometer was attached to the head by clips around the ears and muzzle. The shaft of the potentiometer was directly attached by a lever and universal joint to the suture to allow isotonic transduction of NM movement without a restoring force on the transducer (Gruart and Yeo, 1995). Each subject was placed in a ventilated, sound-attenuating chamber facing a centrally mounted loudspeaker. The conditioned stimulus (CS) was a 1 kHz sine wave tone of 410 ms duration and an intensity of 81 dB (A scale). Background noise produced by ventilation fans was 57 dB (A scale). The unconditioned stimulus (US) was periorbital electrical stimulation (60 ms train of 3 biphasic pulses of intensity 2 mA) through stainless steel clips attached to the skin, one immediately behind the temporal canthus of the eye, the other immediately below the center of the lower eyelid. On paired trials, the interstimulus interval between the CS and US onset was 350 ms. The intertrial interval was randomly selected between 25–35 s.

Habituation Session

Before conditioning training commenced, subjects were allowed a single habituation session (25 min) to adapt to the novel environment of the chamber. During this period, each subject was placed in the restraining stock within the conditioning chamber, and the NM transducer was fitted. The periorbital clips were attached, but the US and CS were not presented.

Conditioning Sessions

Each conditioning session consisted of 50 trials. In 45 trials, the CS and US were paired, and in 5 trials the CS was presented alone. A CS-alone trial was presented on every tenth trial. The acquisition training consisted of five phases with 3 days between each phase. Cortical cannulated subjects were randomly assigned to one of two groups, a muscimol treated group (HVI-M) and a vehicle treated control group (HVI-S). A third group consisted of subjects with cannula implants directed toward the cerebellar nuclei (CN) and received muscimol treatment alone.

Phase 1

All subjects received four daily sessions of acquisition training. Immediately after each session, the HVI-M groups subjects received a cortical infusion of muscimol (3.5 mM, 2 μl in 0.01 M phosphate buffered saline [PBS] [pH 7.4]), and the HVI-S subjects received vehicle (PBS, 2 μl). Each infusion was given over 2 min. CN group subjects received infusions of muscimol into the cerebellar nuclei (7.0 mM, 1 μl in 0.01 M PBS [pH 7.4]) given over 1 min.

Phase 2

All subjects received four daily sessions of training with no posttraining infusions.

Phase 3

Group HVI-M subjects that had received muscimol during Phase 2 received four more daily sessions of training without drug. The HVI-S control subjects received four daily sessions of training, each followed immediately by muscimol infusions to assess the effects of chronic posttraining administration on performance of already established CRs.

Phase 4

The HVI-S subjects were trained for a further four sessions with no drug infusions.

Phase 5

In order to test whether the muscimol infusions in Phase 1 had been in appropriate locations and sufficient fully to inactivate the critical eyeblink control regions, we tested in Phase 5 their efficacy in blocking performance of conditioned responses previously established in Phases 1 and 2. The Phase 5 session began with 20 trials (18 paired CS-US trials and 2 unpaired CS trials). Muscimol (same dose, concentration, and time course as in Phase 1) was then infused. Five minutes after the start of the infusion, the session continued with 20 trials (18 paired CS-US and 2 unpaired CS). Similar blocks of 20 trials were then given at 30 min, 1 hr, 2 hr, 4 hr, 8 hr, and 24 hr after the infusion. In this way, the effects of muscimol infusion were assessed throughout its time to effects and recovery.

Histology

In the final stage of the experiment, [^{3}H]muscimol in PBS (7 nmol in either 1 or 2 μl PBS for cerebellar nuclear and cortical subjects, respectively, containing 1 μCi/μl) was infused through the injection cannulae at the same position as for the previous infusions. This muscimol dose corresponded to the concentration used in the experimental phases. Each subject was then given heparin sodium (500 U/kg, i.v.) and an overdose of pentobarbitone sodium (90 mg/kg, i.v.) 2 hr after the end of the muscimol infusion. This time corresponded to the maximum drug effect upon behavior during Phase 5. Each subject was perfused transcardially with 0.9% saline (1 liter) followed by 4% formaldehyde solution (2 liters). The brain was removed, embedded in gelatin, cryoprotected in 20% sucrose solution, and then serial, 50 μm frozen transverse sections were cut.

Autoradiography and Image Analysis

Every sixth brain section was opposed to tritium-sensitive film (Hyperfilm, Amersham, Uppsala, Sweden) for autoradiography together with tritium standards (Microscales, Amersham) for 6 weeks at 4°C. After film development, the sections were stained with cresyl violet. The autoradiograph of every brain section was imaged with a monochrome CCD camera and analyzed using standard densitometry techniques (AIS, Imaging Research, Canada); the resultant images were calibrated, and their densities were color-coded with reference to the tritium standards as picomoles of muscimol per milligram tissue equivalent (see Figure 4).

An image of each Nissl-stained section was captured and was processed to reveal the brain edges and granule cell layer boundaries. Composites of the color-coded densitometry and the brain contours were then made.

Data Analysis

A CR was defined as a NM response within the CS-US interval with amplitude greater than or equal to 0.5 mm and with onset latency greater than 35 ms from CS onset (see Hardiman and Yeo, 1992). CR frequency (%CRs) was calculated for each block of nine paired trials throughout the conditioning sessions.

Data were analyzed using Kruskal-Wallis one-way ANOVA on ranks tests for main group effects during relevant phases of testing followed, when significant, by Dunn's post hoc multiple comparisons procedure to determine individual changes.

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