A.C.H. Yu, L.F. Eng, K.J. McMahan, H. Schulman. E.M. Shooter and A. Stadlin (Eds.)
Progress in Brain Research, Vol. 105

CHAPTER 1

The gene knockout technology for the analysis of learning and memory, and neural development

Susumu Tonegawa[1], Yuqing Li[1], Reha S. Erzurumlu[4], Sonal Jhaveri[4], Chong Chen[1], Yukiko Goda[3], Richard Paylor[5], Alcino J. Silva[1], Jeansok J. Kim[6], Jeanne M. Wehner[5], Charles F. Stevens[3] and Asa Abeliovich[1]

[1]Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA, [2]Howard Hughes Medical Institute at the Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA, [3]Howard Hughes Medical Institute, Salk Institute, 10010 North Torrey Pines Road, La Jolla, CA 92037, USA, [4]Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, Cambridge, MA 02139, USA, [5]Institute for Behavioral Genetics and School of Pharmacy, University of Colorado, Boulder, CO 80309, USA, and [6]Neuroscience Program, University of Southern California, Los Angeles, CA 90089-2520, USA

Part I. PKCγ mutant mice display altered LTP and mild deficits in spatial learning

Mutant mice produced by embryonic stem (ES) cell gene targeting technique to be defective in a particular gene product provide an attractive model system in which to study long-term potentiation (LTP), the most intensively studied cellular model for memory (see Bliss and Collingridge, 1993). We can obviate the lack of highly specific pharmacological tools to study various enzymes with this approach; furthermore, we can investigate consequences of the mutation for behavior (see for example Silva et al., 1992a,b; Grant et al., 1992). Hippocampal LTP in particular has been suggested to play an important role in certain types of learning and memory and has attracted considerable attention. The availability of knockout mice thus provides a unique opportunity to address the specific role of kinases in LTP, and the relationship between LTP and learning and memory processes.

We have continued a program of investigating the molecular substrates of synaptic plasticity by producing mice that lack the g isoform of Ca^{2+}/phospholipid-dependent protein kinase (PKC) using ES cell gene targeting technology. PKC constitutes a family of isoenzymes involved in signal transduction pathways in diverse systems. This enzyme was chosen for study because pharmacological studies have repeatedly implicated PKC as playing a role in LTP (for reviews see Ben-Ari et al., 1992; Schwartz, 1993). The γ isoform was selected because it is brain specific, is richly represented in hippocampus where LTP and LTD are robustly expressed (Nishizuka, 1988), and appears to be present primarily in the dendrites and cell body of neurons (Huang et al., 1988). Moreover the γ isoform appears late in development, so that brain defects that simply reflect abnormal neural development are less likely.

Physiological analysis of PKCγ mutant mice

Synaptic activity in hippocampal CA1 pyramidal cells is evoked by stimulating Schaffer collateral and commissural axons, and is recorded in two ways: (1) field potential recordings which monitor additive responses from a group of cells, and (2) whole-cell recordings that monitor responses from a single cell. In both field potential and whole-cell recordings, the evoked response in PKCγ mutant slices is indistinguishable from the wild type mice. In whole-cell recordings, the average synaptic current decay time constant for the mutant animals was 11.5 ± 0.5 ms, and for normal animals 11.1 ± 0.3 ms. Because response size depends on stimulus intensity, a comparison of the magnitude of postsynaptic responses is more difficult. We have used the fact that, at sufficiently low stimulus intensities, only a single quantum of transmitter appears to be released most times (Raastad et al., 1992) — so-called "minimal stimulation" — and have compared the response amplitudes and time courses to such minimal stimulation. We could detect no systematic effect of PKCγ absence under minimal stimulation conditions (data not shown).

LTD was produced by the Dudek–Bear protocol (Dudek and Bear, 1992). The mean size, shape of the distribution function and variance of the distribution function are not significantly different for the wild type and mutant animals (LTD was elicited in 26 wild type and 29 mutant slices; data not shown). Thus, LTD is present in the mutant animals that lack PKCγ.

LTP is abnormal

The usual test for LTP is to examine the increase in synaptic strength produced by tetanic stimulation. We have carried out field potential experiments in 14 mutant slices to compare with 17 control slices. LTP was clearly deficient in mutant slices (Fig. 1). Whole-cell recording is a more effective method for producing LTP than field potential recording since it allows a direct control of the postsynaptic membrane potential which is crucial for LTP induction. To test whether LTP deficiency in mutant slices can be overcome by controlling the postsynaptic membrane potential, whole-cell recordings have been carried out (7 control and 12 mutant slices), and similar results were obtained (data not shown). We conclude that LTP is either absent or greatly diminished under the conditions of these experiments.

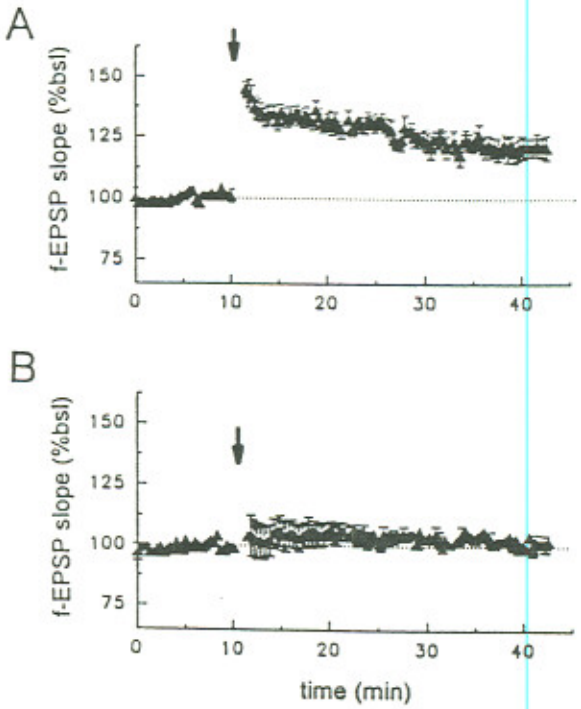

Fig. 1. LTP is abnormal in mutant slices. Summary of field potential recordings from 15 wild type (A; n = 6 mice) and 13 mutant (B; n = 5 mice) slices are shown. Initial slope of field excitatory postsynaptic potentials (f-EPSPs) are expressed as percentage of the mean baseline f-EPSP slope before tetanic stimulation (arrow). The error bars display the standard error of the mean. Tetanus to evoke LTP consisted of 5 trains of 100 Hz stimulation, each lasting 200 ms at an intertrain interval of 10 s. Testing stimuli were given every 20 s. Methods are described elsewhere (Abeliovich et al., 1993a).

Figure 2 displays the summary of recordings in which LTD was elicited prior to the production of LTP. After the LTD protocol, as is clear from this set of experiments, normal appearing LTP can be elicited in the mutant

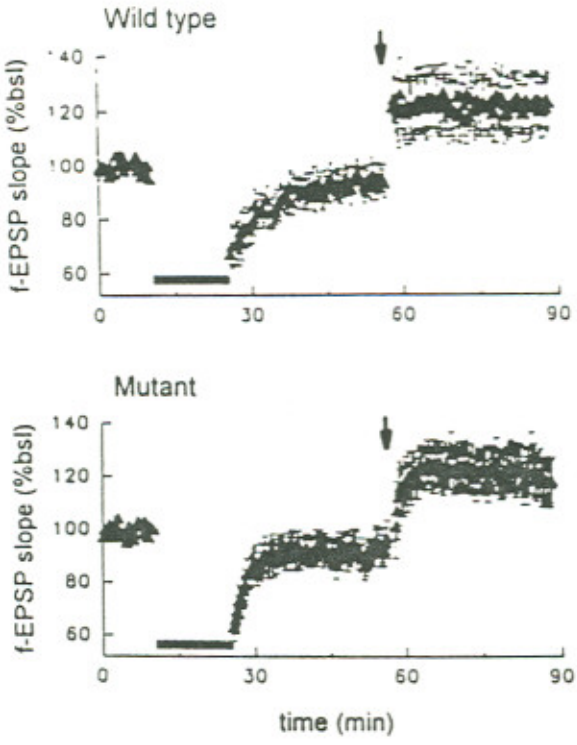

Fig. 2. LTP can be elicited in mutant slices after the LTD protocol. Summary of field potential recordings from 8 wild type (5 mice) and 15 mutant slices (9 mice) where LTD protocol (solid bar) was followed by a tetanus to evoke LTP (arrow). Responses are expressed as % of the baseline response (f-EPSP slope) prior to low frequency stimulation. The error bars display the standard error of the mean. Methods are described elsewhere (Abeliovich et al., 1993a).

slices. When LTP was elicited in the presence of 50 mM AP5 after the LTD protocol no potentiation was observed in the mutant slices; the mean response after the tetanus (81 ± 6%; n = 9) was not significantly different from the mean LTD response (87 ± 3%; n = 29).

The absence of the γ isoform of PKC has no detectable effect on baseline synaptic transmission. Synaptic plasticity is, however, modified but not eliminated because LTD is apparently normal, and LTP — which is absent under the usual test circumstances — can be elicited if preceded by a period of low frequency stimulation.

Behavioral analysis of PKCγ mutant mice

To test the correlation between hippocampal LTP and learning and memory, we subjected PKCγ mutant mice to a spatial learning and memory task, the hidden-platform Morris water maze task, the performance of which is reported to require the hippocampus in rodents. Additionally, we investigated the performance of mutant mice in another task, the visible-platform Morris water maze task that, in rodents, does not require the hippocampus (Morris et al., 1982; Sutherland et al., 1982).

Morris water maze

The Morris water maze (Morris, 1981) consists of a circular pool filled with opaque water and containing an escape platform submerged approximately 1 cm below the surface of the water. In the hidden platform version of the Morris water maze task, mice are placed in the pool at one of 4 start sites, and the platform location is kept constant throughout training. In order to escape the water, mice must learn to navigate to the hidden platform by mapping its position relative to visual cues outside of the pool, a process defined as spatial learning. In the visible-platform Morris water maze task, a cylindrical landmark is placed on the escape platform, indicating its position. Mice are placed in the pool at one of four start sites but, unlike the hidden-platform, the 'visible' platform is relocated to new quadrants of the pool between trials. Therefore, mice must learn to associate the landmark with the location of the platform, and spatial information is irrelevant. In rodents, disruption of NMDA receptor function appears to impair LTP induction as well as performance on the hidden-platform Morris water maze task (Morris et al., 1986), whereas performance on the visible-platform task is unimpaired (Morris et al., 1991). Because the two tasks are similar in terms of motivation and the requirement for swimming ability, the visible-platform task serves as an important control for these factors.

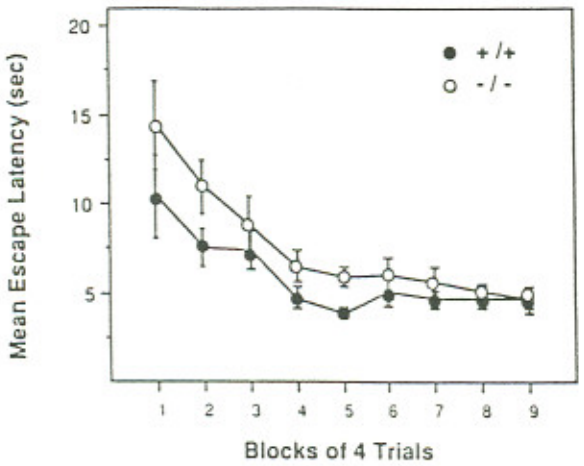

Fig. 3. Average time taken to locate the visible platform (escape latency) for wild-type and PKCγ mutant mice. Mice were trained using a massed-trial (12 trials/day) procedure. These data are from mice previously trained on the hidden-platform task. Performance improved for each genotype [$F(8,104) = 15.547$, $p < 0.0001$], and there was no significant difference between wild-type and mutant mice [$F(1,13) = 2.737$, $p = .122$]. Methods are described elsewhere (Abeliovich et al., 1993b).

Visible-platform Morris water maze task

PKCγ mutant mice were tested in the visible-platform version of the Morris water maze, a non-spatial learning task, following training on the hidden-platform task. The performance of PKCγ mutant mice (n = 8) was not significantly different ($p = 0.122$) from that of wild-type mice (n = 7), although mutant mice tended to perform somewhat more poorly that wild-type mice initially (Fig. 3). In the course of training, mutant mice did reach the wild-type level of performance, demonstrating that mutant mice can learn this task, and suggesting that the initial impairment displayed by mutant mice does not prevent them from learning. Similar results were observed when the mice were not trained on the hidden-platform task prior to visible-platform training (data not shown).

Hidden-platform Morris water maze task

Spatial learning was tested in the hidden-platform version of the Morris water maze task. Both mutant (n = 12) and wild-type (n = 9) mice displayed significant improvement over the 9 blocks of training ($p < 0.0001$), and the two groups did not differ significantly ($p = 0.598$) (Fig. 4A). Mice can improve their performance in the hidden-platform task by adopting a learning strategy other than spatial learning. For instance, the mice may learn that the platform is located a certain distance away from the edge of the pool. This strategy is not as precise as the spatial learning strategy, but it nevertheless enables mice to find the platform more quickly than a random search does.

In order to confirm that the PKCγ mutant mice used a spatial learning strategy, we subjected the trained mice to a probe test in which the platform was removed and the mice were allowed to search the pool for 60 s. Both mutant ($p < 0.0001$) and wild-type ($p < 0.0001$) mice selectively searched the quadrant in which the platform had been located during the training versus all other quadrants (Fig. 4B). Furthermore, both mutant ($p < 0.0001$) and wild-type ($p < 0.0001$) mice crossed the exact location at which the platform had been located during training more frequently than any of the corresponding locations in the other quadrants (Fig. 4C). However, in this platform site-crossing test, mutant mice did cross the correct site less often than wild-type mice ($p < 0.05$), indicating that mutants harbor a moderate deficit in spatial learning.

Conclusion

Hippocampal LTP is abnormal in PKCγ mutants in that it is absent or greatly attenuated when induced *in vitro* by conventional tetanic stimulation, although apparently normal LTP can be enabled by prior low-frequency stimulation. Thus, our overall data show that LTP, as assessed by conventional tetanic stimulation, is not essential for mice to exhibit hippocampus-dependent learning. However, the modified properties of hippocampal LTP correlate with mild to moderate deficits in spatial learning, consistent with the notion that LTP is a

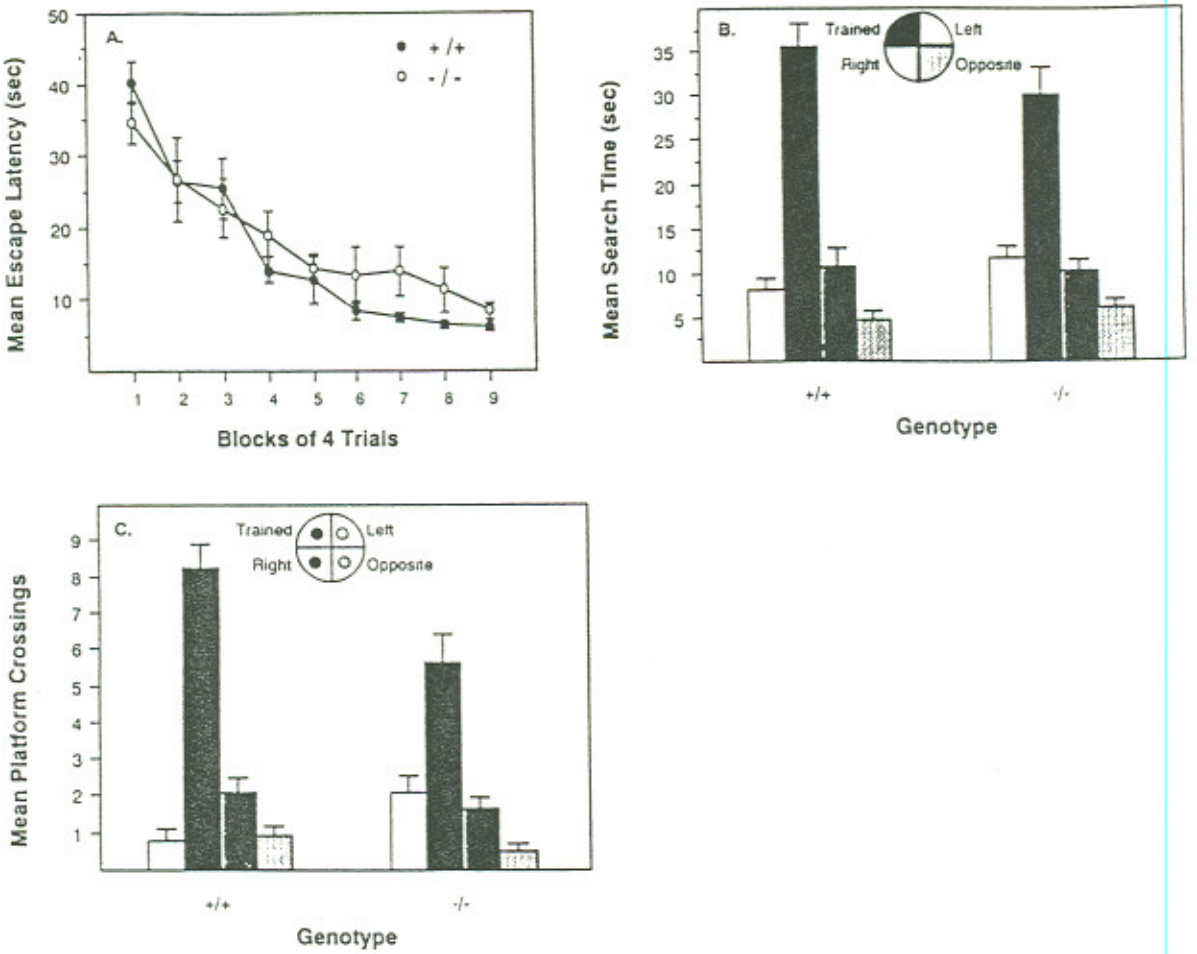

Fig. 4. Performance of wild-type and PKCγ mutant mice trained on the hidden-platform version of the Morris water task. Mice were trained using a distributed-trial (4 trials/day) procedure. (A) Average escape latency during training. Performance of wild-type and mutant mice improved during training [$F(8,152) = 34.695$, $p < 0.0001$], and there was no difference between the two genotypes [$F(1,19) = 0.286$, $p = 0.5987$]. (B) Average time subjects spent in each quadrant of the pool during the probe test. (C) Mean platform crossings during the probe trial. Wild-type mice spent more time in the training quadrant compared to the other quadrants [$F(3,24) = 44.777$, $p < 0.0001$. Newman–Keuls post-hoc comparison: Trained > All other quadrants, $p < 0.01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,24) = 57.517$, $p < 0.0001$. Newman–Keuls post-hoc comparison: Trained > All other quadrants, $p < 0.01$]. Similarly, mutant mice spent more time in the training quadrant than the other quadrants [$F(3,33) = 20.988$, $p < 0.0001$. Newman–Keuls post-hoc comparison: Trained > All other quadrants, $p < 0.01$] and crossed the site where the platform was located more often than the alternate sites [$F(3,33) = 13.251$, $p < 0.0001$. Newman–Keuls post-hoc comparison: Trained > All other quadrants, $p < 0.01$]. Wild-type mice did not spend any more time in the training quadrant compared to mutant mice [$t(19) = 1.145$, $p > 0.26$], but did cross the correct site more often than the mutants [$t(19) = 2.337$, $p < 0.031$]. Methods are described elsewhere (Abeliovich et al., 1993b).

synaptic mechanism for this form of learning. The learning deficit observed in PKCγ mutant mice may be causally related to the LTP modification.

Another interesting candidate synaptic mechanism for learning that has emerged from this and other recent studies is LTD. While there has been no direct evidence, LTD satis-

fies the same criteria for a synaptic learning mechanism as does LTP (Siegelbaum and Kandel, 1991). Furthermore, LTD appears to correlate with spatial learning capability, as it is intact in PKCγ mutant mice (Abeliovich et al., 1993), which display spatial learning, whereas it is impaired in αCaMKII mutant mice (C. F. Stevens, S.T., and Y. Wang, unpublished observations), which are deficient in spatial learning (Silva et al., 1992b). It is also of note that AP5, which has been shown to impair spatial learning (Morris et al., 1986), is now known to block not only LTP but also LTD (Dudek and Bear, 1992; Mulkey and Malenka, 1992). It is therefore possible that the learning impairment observed in AP5 treated animals results from the disruption of both LTP and LTD.

Part II: Whisker-related neuronal patterns fail to develop in the trigeminal brainstem nuclei of NMDAR1 knockout mice

In most sensory systems, the periphery is represented in a topographic fashion in the brain. Studies of the mechanisms involved in the development of such maps have focused on the visual system, and have implicated a key role for NMDA receptor-mediated activity in the refinement of topographic maps (for reviews see Constantine-Paton et al., 1990; Shatz, 1990). The prevailing hypothesis states that correlated activity in afferent which converge on specific neurons results in depolarization of the postsynaptic membrane via non-NMDA receptors and removal of the magnesium block on NMDA receptors. This allows the glutamate released by subsequent action potentials to open the NMDA receptor channel, Ca^{++} enters into the postsynaptic cell, and triggers the activation of unknown downstream events in providing feedback to the presynaptic fibers, leading to consolidation of synaptic connections which fire in synchrony. In contrast, connections which do not fire in synchrony are weakened.

Attempts to generalize these finding to somatosensory system, in particular the rodent whisker-to-barrel system, have generated mixed results. Although lesions of the sensory periphery within a few days of birth dramatically alter whisker-related patterns in the brain (Belford and Killackey, 1980; Jeanmonod et al., 1981; Durham and Woolsey, 1984), activity blockade experiments failed to prevent the emergence of these patterns (Chiaia et al., 1992b; Henderson et al., 1992; Schlaggar et al., 1993). These led to the suggestion that the development of whisker-related patterns may be established independent of activity (Katz, 1993; Purves et al., 1994).

In order to re-examine the role of NMDA receptor-mediated activity in the establishment of neural patterns in the whisker-to-barrel system, we have used 'reverse genetics' to selectively 'knock out' (reviewed in Capecchi, 1989) the NMDAR1 subunit of the NMDA receptor. Our results show that in the knockout animals, although central targeting and topographic projection of the trigeminal afferent appear to be normal and postsynaptic neurons are responsive to stimulation of primary trigeminal afferent, whisker-specific neural patterns fail to develop in the absence of the NMDA receptor.

NMDA Receptor-deficient mice

NMDAR1 subunit is essential for NMDA receptor activity (reviewed in Nakanishi, 1992). The NMDAR1 targeting vector replaced a 2.4 kb region encoding four transmembrane domains by a neomycin-resistance gene upon homologous recombination in embryonic stem (ES) cells. ES cells of the targeted clones were injected into blastocysts, which were then implanted into foster mothers (Bradley, 1987) to get chimeric animals. Chimeric animals transmitted the mutation to their offspring. Heterozygous animals (F1) were bred to produce homozygous mutants (F2).

The mutant animals die before P2. The mutant animals can not suckle milk. Moreover,

the mutant animals are severely ataxic and cannot support their bodyweight on their hindlimbs. No abnormality was detected in the electrocardiographic pattern or the respiratory rhythm of newborn mutant mice. However, increased apnea is observed within several hours of birth and cyanosis appears shortly before death, suggesting that respiratory failure contributes to mutant animal fatality. The average bodyweight for newborn mutant pups is close to that of normal pups indicating that the embryonic development of the knockout mice is not significantly perturbed.

Whisker-related patterns are absent in NMDAR1 mutant mice

In the brain of the normal mouse, the five rows (A–E) of whiskers are represented by discrete five rows of neuronal modules in brainstem trigeminal nuclei (BSTC), ventrabasal thalamus and primary somatosensory cortex (reviewed in Woolsey, 1990). The whisker-specific modules at BSTC level (barrelettes) can be visualized by staining for cytochrome oxidase (CO). Mice pups are born between E18.5 and E19.5 (we refer to the morning of E19 as P0). In normal animals, CO histochemistry reveals an emerging segmentation of the reaction product at the earliest time (E18.5) that the mice are born. In all our wild type (n = 4) and heterozygous animals (n = 3), individual barrelettes are readily discernible by late P0 (E19.5). However, in none of the mutant animals (n = 9), is there any indication of row or patch formation. In order to rule out the possibility of developmental retardation in the mutant animals, the birth of several litters was blocked for one day. In combination, we attempted to extend the life of the mutant animals by CO_2 stimulation after birth. Normal littermate pups were treated the same way as controls. These measures allowed the mutants to survive up to 21 days after conception (equivalent to P2; n = 3). These mutant mice failed to even form rows of barrelettes (Fig. 5). The lack of barrelette formation in knockout animals was confirmed with the use of an alternative marker: cytotactin-binding proteoglycan (CTBP, Fig. 6). CTBP is an extracellular matrix molecule secreted by neurons. These observations confirm that barrelettes do not form in the mice which lack functional NMDA receptors.

The gross appearance of the whiskers and their arrangement into five rows (A–E) is normal in the newborn mutant mice. In order to determine that the absence of barrelettes does not result from a delayed connectivity between the whisker pad and the BSTC, we compared the development of the peripheral trigeminal pathway in control and mutant animals. Immunohistochemical staining using an antibody (TuJ1) against neuron-specific tubulin in E11 embryos revealed that trigeminal axons reach their peripheral and central targets at the same time both in mutants (n = 4) and in controls (n = 6; cf. Stainier and Gilbert, 1990), and that there are no gross differences in the size of the trigeminal ganglion, or in the density of its projections, between the two types of animals. Next, we applied non-overlapping crystals of the lipophilic tracer DiI in the dorsal (A) and ventral (E) whisker rows on one side of the snout, and in the middle whisker row (C) on the opposite side, in normal (n = 12) and mutant mice (n = 12) that were killed and fixed on E17 or on P0 (Erzurumlu and Jhaveri, 1992). The results of the labeling suggest that the topographic ordering is present in the central trigeminal projections of both mutant and normal mice.

Excitability of brainstem trigeminal neurons and synaptic transmission

We performed *in situ* hybridization to further investigate the role of NMDA receptors in the formation of barrelettes. The results indicate that the NMDAR1 mRNA is present in the BSTC of normal newborn mice. To study whether the mRNA is translated and forms functional NMDA receptors, we used whole-cell patch clamp recording. Cells within the whisker representation region of the BSTC slices

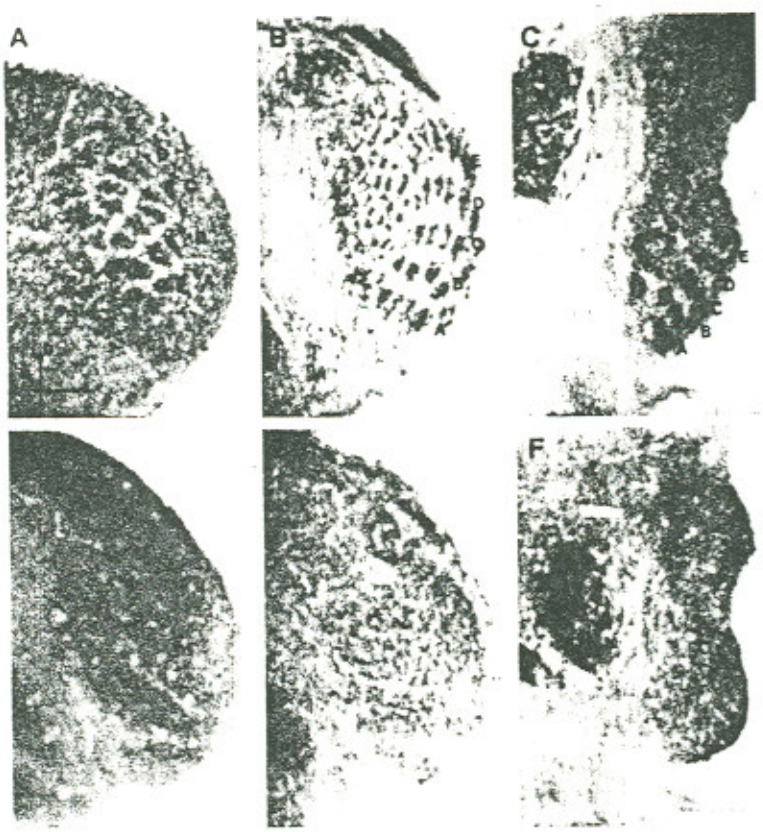

Fig. 5. Cytochrome oxidase-stained sections in wild type (top row) and mutant animals (bottom row), both aged P1.5. Sections are shown at the level of subnucleus caudalis (A, D), and interpolaris (B, E) of the spinal trigeminal nucleus, and also at the level of nucleus principalis (C, F). Individual barrelettes are visible within the 5 whisker row representations (A–E) at all three levels of the brainstem trigeminal complex in the normal animal, whereas they are lacking at all three levels in the knockout mouse. MV: motor nucleus of the trigeminal nucleus. Cytochrome oxidase staining in MV is dense, both in control and mutant animals, indicating that failure of pattern formation in the mutants is not merely a reflection of the general absence of cytochrome oxidase activity throughout the brain. D: dorsal, L: lateral for all micrographs. Scale: 0.2 mm for all six micrographs. Aldehyde-fixed, cryoprotected brainstems were cut into 50 m thick sections in the coronal plane on a freezing microtome. Cytochrome oxidase histochemistry was performed according to the procedure described by Wong-Riley (1979).

were identified as neurons based on their neuronal excitability (Fig. 7A). Under the voltage-clamp, electrical stimulation of the trigeminal tract routinely evoked excitatory postsynaptic currents (EPSCs; Fig. 7B,C). In mutant slices (n = 3), only a fast inward (CNQX-sensitive) current was apparent, indicating the absence of NMDA receptor-mediated component (Fig. 7B). In wild-type slices (n = 2), there were both a fast non-NMDA receptor-mediated (sensitive to CNQX) and a slow NMDA receptor-mediated (sensitive to AP5) postsynaptic currents (Fig. 7C), suggesting that the transmission is mediated via glutamate in this pathway. It is also important to note that inhibitory synaptic currents (IPSCs) were present in some mutant cells (Fig. 7D).

Discussion

Studies on the role of NMDA receptors during map formation have used primarily NMDA receptor antagonists, we have used gene tar-

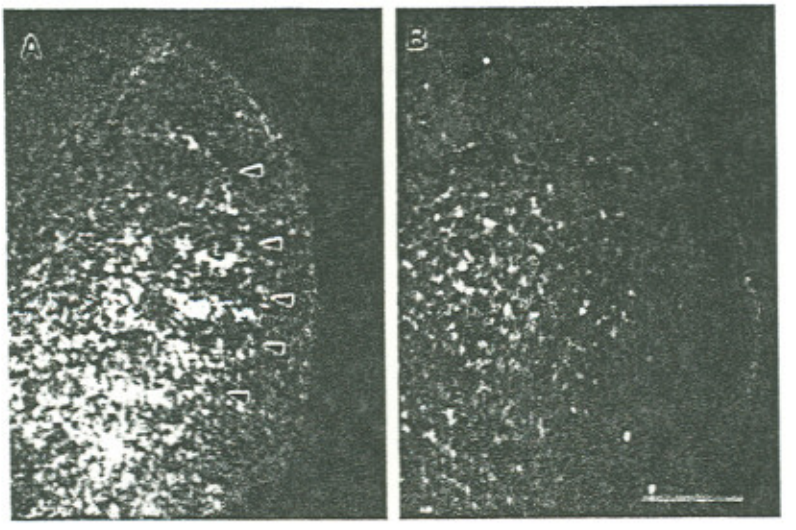

Fig. 6. Barrelettes detected by immunohistochemistry for CTBP. Coronal sections through the brainstem of a normal (A) and a mutant (B) P0 mice at the level of subnucleus interpolaris. The sections were immunostained with an antibody against the extracellular matrix molecule cytotactin-binding proteoglycan. The immunopositive pattern in the brainstem of the normal control animal reflects the emerging pattern of barrelettes. Note that the proteoglycan is deposited in the regions between rows. The five whisker-specific rows are indicated by arrowheads. No emergent pattern can be discerned with this antibody along the BSTC of the mutant animal. Scale: 0.2 mm for both micrographs. Immunohistochemistry for cytotactin-binding proteoglycan (CTBP) was carried out according to published protocols (Crossin et al., 1989; Jhaveri et al., 1991; Easter et al., 1993).

geting to eliminate the gene which encodes an essential subunit of the NMDA receptor. Results from whole cell patch clamp recording of brainstem trigeminal neurons in the mutant mice confirmed that we have "knocked out" NMDA receptor activity (Fig. 7B). Our results show that in the absence of NMDA receptors and in the presence of functional excitatory and inhibitory synaptic transmission, postsynaptic neurons fail to aggregate into barrelettes as detected by two independent staining methods: CO histochemistry and CTAB immunohistochemistry. Furthermore, trigeminal sensory axons form topographically appropriate connections between the whisker pad and the BSTC along the normal time schedule in the mutants. We thus conclude that NMDA receptors are involved in the detailed patterning of target neurons, and most likely of afferent axons as well, to reflect the precise arrangement of peripheral sensory organs.

Studies in which TTX or AP5 was applied to the infraorbital nerve or to the developing somatosensory cortex of rats did not result in blocking the formation of barrel patterns in the somatosensory cortex (Chiaia et al., 1992b; Henderson et al., 1992; Schlaggar et al., 1993). These reports led to the general belief that in the trigeminal system, activity plays a less important role during the development of whisker-related patterns (Katz, 1993; Purves et al., 1994). However, it should be noted that in the above experiments, activity was blocked as of the day of birth. In the BSTC of the rat the whisker-related pattern is present before the time of birth (Chiaia et al., 1992a) and in some strains of rats, thalamocortical axons are already patterned on P0 (Schlaggar and O'Leary, 1993). Thus organizational information from the BSTC may already have been encoded in the developing thalamocortical axons by the time activity or NMDA receptor blockade is

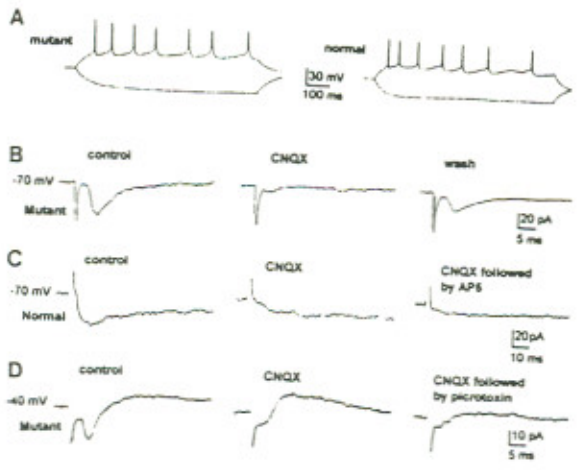

Fig. 7. Neuronal excitability and synaptic transmission are present in the BSTC of P0 mutant and normal mice. (A) Membrane voltage changes in response to current steps (±0.04 nA). The depolarizing current step initiated a train of action potentials in both the mutant and normal neurons. (B) The synaptic current evoked by trigeminal tract stimulation has only non-NMDA receptor-mediated component in the mutant slice. The fast inward current evoked at a holding potential of –70 mV was abolished by CNQX (15 M) and recovered after subsequent washout of CNQX. (C) The synaptic current has both NMDA and non-NMDA receptor-mediated components in the normal slice. After application of CNQX (15 M), a small slow inward current remained and was later abolished by the addition of AP5 (100 M). Holding potential, –70 mV. (D) The inhibitory synaptic current was present in the mutant slice. The synaptic current evoked at a holding potential of –40 mV has a fast, inward non-NMDA receptor-mediated component and a slow, outward $GABA_A$ receptor-mediated component. The latter was blocked by picrotoxin (100 M). Methods: Animals (P0) were decapitated under Metofane anesthesia, the brain was removed rapidly and placed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) composed of (in mM): 119 NaCl, 2.5 KCl, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4, 10 glucose (pH 7.4). Coronal slices, 500 m thick, were cut with a vibratome through the brainstem, then maintained in an incubation chamber for at least one hour at room temperature (22–25°C). For recording experiment, a slice was transferred to a submerge-recording chamber where it was held by a nylon net and constantly perfused with oxygenated ACSF (95% O_2 and 5% CO_2). Whole-cell recordings were obtained with conventional patch techniques. Patch electrodes (4–8 M) were fabricated from soft glass (Drummond), pulled on a three-step puller (Sutter Instrument). The internal solution contained (in mM) K acetate (90), KCl (20), $MgCl_2$ (2), EGTA (3), HEPES (40), $CaCl_2$ (1), ATP-Mg (2), and GTP-Li (0.2); pH 7.2. Once into the whole-cell mode, current injection experiments were first performed under the current-clamp. Resting membrane potentials ranged from –45 to –65 mV. A constant holding current was used to keep the resting potential at –60 mV if necessary. Synaptic currents were then recorded under the voltage-clamp. Trigeminal fibers were stimulated by a concentric bipolar electrode (Rhodes Instrument) that delivered 0.1 ms pulses. Data were digitized, stored and analyzed on a 486 computer with a analog to digital converter (Digidata) and pCLAMP program (both by Axon Instrument). Signals were filtered at 2 KHz. Synaptic current records shown were the average of six traces sampled at an interval of 20 s. Drugs were applied via bath application at final concentrations of 15 M CNQX (Tocris Neuramin), 100 M AP5 (Tocris Neuramin), or 100 M picrotoxin (Sigma).

initialized. Our results document that in the absence of functional NMDA receptors, barrelettes fail to develop in the hindbrain. Extrapolating from this result, we suggest that the formation of whisker-related patterns (barreloids and barrels) in the thalamus and cortex might also depend on activation of NMDA receptors.

We have demonstrated that in the absence of NMDA receptors, brainstem trigeminal neurons are excitable and both excitatory and inhibitory synaptic transmission is functional in the mutants (Fig. 7). Thus sensory inputs from the periphery could be transmitted to and processed in both the mutant and normal BSTC. We hypothesize that the failure of the barrelette formation is not caused by the lack of neuronal excitability and synaptic transmission in the BSTC of NMDAR1 mutants but by the blockade of the downstream cascade initiated by NMDA receptor activation. The availability of this mutant mice will greatly help us to elucidate the detailed pathway following NMDA receptor activation during map formation in the mammalian central nervous system.

Acknowledgements

Part I: This work was supported by the Howard Hughes Medical Institute (S.T. and C.F.S.), the Shionogi Institute for Medical Science (S.T.), the National Institutes of Health grant RO1 NS 12961-17 (C.F.S.), the National Institute of Mental Health grant MH-48663 (J.M.W.), and a National Institute of Alcohol Abuse and Alcoholism Research Scientist Development Award AA-00141 (J.M.W.). A.A. is a predoctoral fellow of the National Institutes of Health Medical Scientist Training Program. Y.G. is supported by a Damon Runyon-Walter Winchell Cancer Research Fund Fellowship DRG-1104.

Part II: We wish to thank Drs. K. Crossin, A. Frankfurter, R. Kemler, M. Rudnicki, A.J. Silva for their gift of various reagents, Sang Hsu, Wei Lin and Ekaterina Golland for technical assistance, Drs. Luc Van Kaer and Alcino J. Silva for help with ES cell work, Drs. Philip G. Ashton-Rickardt and En Li for the advice on making chimeras, Dr. Barry Kosofsky for help with in situ hybridization, Dr. Donald G. Rainnie for advice on brainstem recording and the members of Tonegawa lab for helpful discussion and encouragement. This work was funded by the HHMI (S.T.), the Shionogi Institute for Medical Science (S.T.), and by a grant from the National Institute of Health (NS 27678) to S.J.

References

Abeliovich, A., Chen, C., Goda, Y., Stevens, C. and Tonegawa, S. (1993a) Modified hippocampal long-term potentiation in PKCγ mutant mice. *Cell*, 75: 1253–1262.

Abeliovich, A., Paylor, R., Chen, C., Kim, J., Wehner, J. and Tonegawa, S. (1993b) PKCγ mutant mice exhibit moderate deficits in contextual learning. *Cell*, 75: 1263–1272.

Belford, G.R. and Killackey, H.P. (1980) The sensitive period in the development of the trigeminal system of the neonatal rat. *J. Comp. Neurol.*, 193: 335–350.

Ben-Ari, Y., Aniksztejn, L. and Bregestovski, P. (1992) Protein kinase C modulation of NMDA currents: an important link for LTP induction. *Trends Neurosci.*, 15: 333–339.

Blue, M.E., Erzurumlu, R.S. and Jhaveri, S. (1991) A comparison of pattern formation by thalamocortical and serotonergic afferents in the rat barrel field cortex. *Cerebral Cortex*, 1: 380–389.

Bradley, A. (1987) Production and analysis of chimeric mice. In: E.J. Robertson (Ed.), *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*. IRL Press, Oxford, pp. 113–151.

Capecchi, M.R. (1989) Altering the genome by homologous recombination. *Science*, 244: 1288–1292.

Chiaia, N.L., Bennett-Clarke, C.A. and Rhoades, R.W. (1992a) Differential effects of peripheral damage on vibrissa-related patterns in trigeminal nucleus principalis, subnucleus interpolaris, and subnucleus caudalis. *Neuroscience* 49: 141–156.

Chiaia, N.L., Fish, S.E., Bauer, W.R., Bennett-Clarke, C.A. and Rhoades, R.W. (1992b) Postnatal blockade of cortical activity by tetrodotoxin does not disrupt the formation of vibrissae-related patterns in the rat's somatosensory cortex. *Dev. Brain Res.*, 66: 244–250.

Constantine-Paton, M., Cline, H.T. and Debski, E. (1990) Patterned activity, synaptic convergence, and the NMDA receptor in the developing visual pathways. *Annu. Rev. Neurosci.*, 13: 129–154.

Crossin, K.L., Hoffman, S., Tan, S.S. and Edelman, G.M. (1989) Cytotactin and its proteoglycan ligand mark structural and functional boundaries in somatosensory cortex of the early postnatal mouse. *Dev. Biol.* 136: 381–392.

Dudek, S.M. and Bear, M.F. (1992) Homosynaptic long-term depression in area CA1 of hippocampus and effects of N-methyl-D-aspartate receptor blockade. *Proc. Natl. Acad. Sci. USA*, 89: 4363–4367.

Durham, D. and Woolsey, T.A. (1984) Effects of neonatal whisker lesions on mouse central trigeminal pathways. *J. Comp. Neurol.*, 223: 424–447.

Easter, S.S. Jr., Ross, L.S. and Frankfurter A. (1993) Initial tract formation in the mouse brain. *J. Neurosci.*, 13: 285–299.

Erzurumlu, R.S. and Jhaveri, S. (1992) Trigeminal ganglion cell processes are spatially ordered prior to the differentiation of the whisker pad. *J. Neurosci.*, 12: 3946–3955.

Grant, S.G., O'Dell, T.J., Karl, K.A., Stein, P.L., Soriano, P. and Kandel, E.R. (1992) Impaired long-term potentiation, spatial learning, and hippocampal development in fyn mutant mice. *Science*, 258: 1903–1910.

Henderson, T.A., Woosley, T.A. and Jacquin, M.F. (1992) Infraorbital nerve blockade from birth does not disrupt central trigeminal pattern formation in the rat. *Dev. Brain Res.*, 66: 146–152.

Huang, F.L., Yoshida, Y., Nakabayashi, H., Young, W.S. and Huang, K.-P. (1988) Immunocytochemical localiza-

tion of protein kinase C isozymes in rat brain. *J. Neurosci.*, 8: 4734–4744.

Jeanmonod, D., Rice, F.L. and Van der Loos, H. (1981) Mouse somatosensory cortex: alterations in the barrelfield following receptor injury at different early postnatal ages. *Neuroscience*, 6: 1503–1535.

Jhaveri, S., Erzurumlu, R.S. and Crossin, K. (1991) Barrel construction in rodent neocortex: role of thalamic afferents versus extracellular matrix molecules. *Proc. Natl. Acad. Sci. USA*, 88: 4489–4493.

Katz, L.C. (1993) Cortical space race. *Nature*, 364: 578–579.

Morris, R.G.M. (1981) Spatial localization does not require the presence of local cues. *Learn. Motiv.*, 12: 239–260.

Morris, R.G.M., Garrud, P., Rawlins, J.N.P. and O'Keefe (1982) Place navigation impaired in rats with hippocampal lesions. *Nature*, 297: 681–683.

Morris, R.G.M., Anderson, E., Lynch, G.S. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by an N-methyl-D-aspartate receptor antagonist AP5. *Nature*, 319: 774–776.

Morris, R.G.M., Davis, S., Butcher, S.P. (1991) Hippocampal synaptic plasticity and N-methyl-D-aspartate channels: a role in information storage? In: M. Baudry and J.L. Davis (Eds.), *Long-Term Potentiation: A Debate of Current Issues*. MIT Press, Cambridge, MA.

Mulkey, R.M. and Malenka, R.C. (1992) Mechanisms underlying induction of homosynaptic long-term depression in area CA1 of the hippocampus. *Neuron*, 9: 967–975.

Nakanishi, S. (1992) Molecular diversity of glutamate receptors and
implications for brain function. *Science*, 258: 597–603.

Nishizuka, Y. (1988) The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature*, 334: 661–665.

Purves, D., Riddle, D.R., White, L.E. and Gutierrez-Ospina, G. (1994) Neural activity and the development of the somatic sensory system. *Curr. Opin. Neurobiol.*, 4: 120–123.

Raastad, M., Storm, J.F. and Andersen, P. (1992) Putative single quantum and single fibre excitatory postsynaptic currents show similar amplitude range and variability in rat hippocampal slices. *Eur. J. Neurosci.*, 4: 113–117.

Schlaggar, B.L., Fox, K. and O'Leary, D.D.M. (1993) Postsynaptic control of plasticity in developing somatosensory cortex. *Nature*, 364: 623–626.

Schlaggar, B.L. and O'Leary, D.D.M. (1993) Patterning of the barrel field in somatosensory cortex with implications for the specification of neocortical areas. *Perspect. Develop. Neurobiol.*, 1: 81–92.

Schwartz, J.H. (1993) Cognitive kinases. *Proc. Natl. Acad. Sci. USA*, 90: 8310–8313.

Shatz, C.J. (1990) Impulse activity and the patterning of connections during CNS development. *Neuron*, 5: 745–756.

Siegelbaum, S.A. and Kandel, E.R. (1991) Learning-related synaptic plasticity: LTP and LTD. *Curr. Opin. Neurobiol.*, 1: 113–120.

Silva, A., Stevens, C.F., Tonegawa, S. and Wang, Y. (1992a) Deficient hippocampal long-term potentiation in a-calcium-calmodulin kinase II mutant mice. *Science*, 257: 201–206.

Silva, A., Paylor, R., Wehner, J. and Tonegawa, S. (1992b) Impaired spatial learning in a-calcium-calmodulin kinase II mutant mice. *Science*, 257: 206–211.

Stainier, D.Y.R. and Gilbert, W. (1990) Pioneer neurons in the mouse trigeminal sensory system. *Proc. Natl. Acad. Sci. USA*, 87: 923–927.

Sutherland, R.J., Kolb, B. and Wishaw, I.Q. (1982) Spatial mapping: definitive disruption by hippocampal or medial frontal cortical damage in the rat. *Neurosci. Lett.*, 31: 271–276.

Wong-Riley, M. (1979) Changes in the visual system of monocularly sutured or enucleated cats demonstrable with cytochrome oxidase histochemistry. *Brain Res.*, 171: 11–28.

Woolsey, T.A. (1990) Peripheral alteration and somatosensory development. In: E.J. Coleman (Ed.), *Development of Sensory Systems in Mammals*. Wiley, New York, pp. 461–516.