Synaptic Activity and the Formation and Maintenance of Neuronal Circuits

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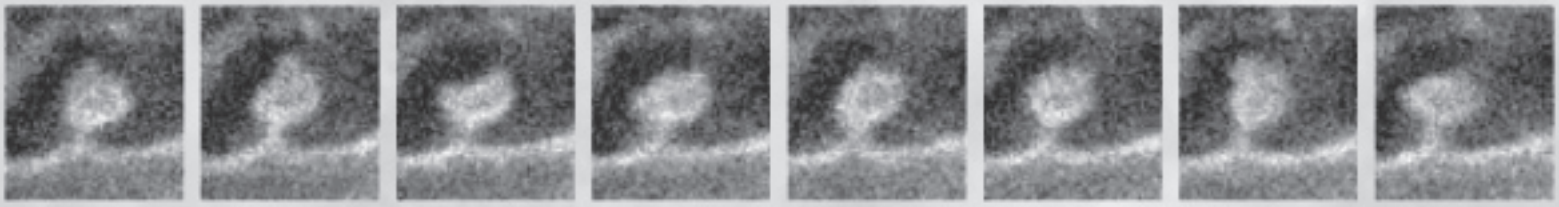
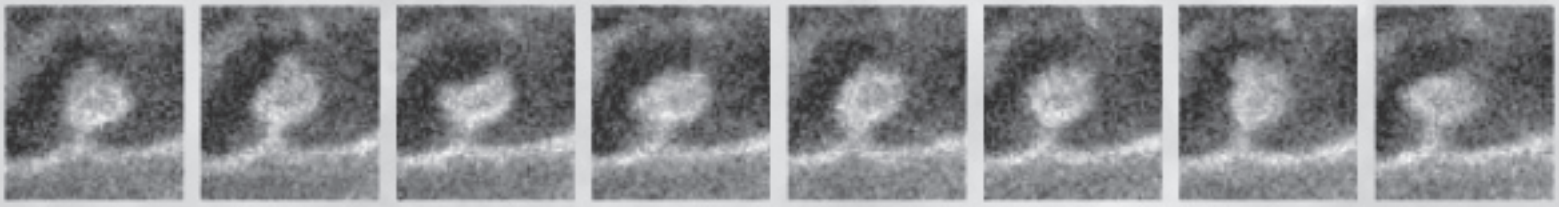


SUMMARY



One of the most fundamental features of neurons is their polarized organization with two types of neurites extending from the cell body, axons and dendrites that are both functionally and morphologically distinct. During development, both axons and dendrites possess highly dynamic and actin-rich growth cones and filopodia extending from their shafts, which are subsequently replace by fundamentally stable axonal varicosities and dendritic spines. Together they form the basic elements of mature synapses.

To mimic in vivo neuronal development, I have used organotypic cultures of brain tissue from transgenic mice expressing either green fluorescent protein (GFP) bearing a surface membrane localization signal or actin-GFP in combination with live cell imaging system. This approach provided me with high-resolution images of developing neurons' fine structure in organized tissue. Co-cultures of fluorescent and non-fluorescent hippocampal slices enabled me then to examine simultaneously dendrite differentiation in the fluorescent slice and to track the fate of fluorescent axons growing into the non-fluorescent slice. Together this granted me a powerful tool to study neuronal network formation and developmental maturation of axons and dendrites.

Co-cultures of embryonic tissue showed a sustained cross-innervation of axonal projections. Over time neurons in these co-cultures formed a dense axonal network with numerous axonal varicosities along their shaft. This axonal plexus remained present beyond 2 months in vitro. Dendrites in these embryonic co-cultures subsequently switched from producing labile filopodia to fundamentally stable dendritic spines. These mature dendritic spines had morphologies similar to those reported from studies of adult brain. Both axons and dendrites exhibited a successive focalisation of actin-based dynamics to the site of the synaptic junction. The observed changes in shape of mature axonal varicosities and dendritic spines together with the rapidly extension and retraction of actin-rich protrusions from the top of varicosities and spine heads suggest a retained capacity for experience-dependent fine-tuning e.g. during either periods of learning and memory or during brain damage resulting in an altered connectivity for both pre- and postsynaptic compartments in the mature mammalian central nervous system. The observed morphological dynamics suggest a high degree of preservation of morphological plasticity at the synapse in mature neuronal networks.

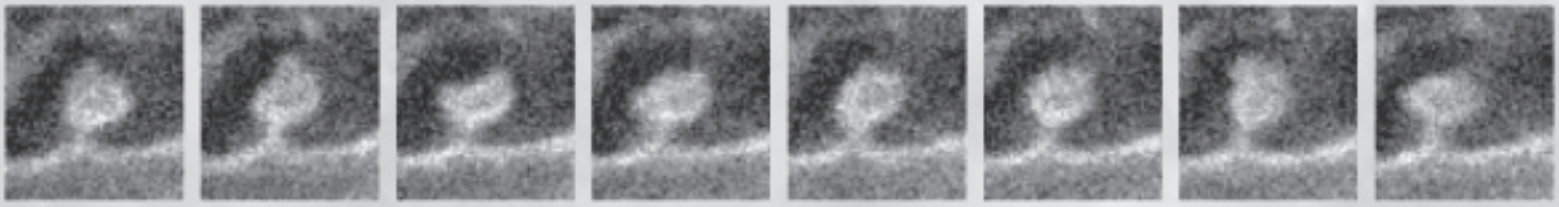
Co-cultures of postnatal brain slices showed intensive invasion of axonal projections during the first two weeks in culture, followed by dramatic axonal regression and resulting in a near complete absence of cross-innervating axons after 1 month in vitro. In contrast, dendrite development in each of these postnatal cultures was fundamentally normal and occurred similar to that observed in embryonic co-cultures. I then co-cultured embryonic and postnatal slices to investigate whether the difference in capacity to cross-innervate between postnatal co-cultures and embryonic co-cultures were the result of tissue maturation. We found that the postnatal slice degenerated so that after 1 month in culture it had almost disappeared whereas the neighbouring embryonic slice had matured without noticeable problems. Staining these co-cultures of embryonic and postnatal slices showed a massive invasion of microglial cells into the dying postnatal slice.

The difference between embryonic and postnatal neurons in their capacity to maintain cross-innervating synaptic connection suggests the existence of a developmental switch resulting in the inability of sustained afferent cross-innervation between postnatal brain slices. At the same time, in heterochronic co-cultures it causes miscommunication between postnatal and embryonic cells leading to profound degeneration of postnatal tissue. The thick layer of microglia surrounding postnatal tissue suggests their involvement in neuronal degeneration similar to that observed in axotomy-induced neuronal death and various neurodegenerative conditions such

as Alzheimer's disease.

The earlier suggested preservation of morphological plasticity at the synapse in mature neuronal networks was illustrated by cooling mature hippocampal slices, either acutely cut brain slices or organotypic cultures, to room temperature. Dendritic spines are highly sensitive to reduced temperature with rapid loss of actin-based motility followed by disappearance of the entire spine structure within 12 hours. However, rewarming these cooled slices to 37˚C resulted in the rapid extension of filopodia from the surface of dendrites and re-establishment of dendritic spines within several of hours. These data underline the high degree of plasticity retained by neuronal connections in the mature CNS and suggest a link between dendritic spine structure and global brain function.

SAMENVATTING



Tijdens de embryonale ontwikkeling van onze hersenen vormen de zenuwcellen talloze onderlinge verbindingen waardoor ze in een later stadium intensief met elkaar kunnen communiceren. In het volwassen stadium verloopt deze communicatie via twee soorten van uitlopers te weten axonen, die signalen afgeven aan andere zenuwcellen, en dendrieten, die dezelfde signalen ontvangen en geleiden naar de zenuwcel. Naast dit verschil in functie verschillen deze twee uitlopers ook in vorm.

Vroeg in de ontwikkeling zien beide uitlopers er anders uit dan in de volwassen hersenen. In het begin stadium van de ontwikkeling vormen zowel axonen als dendrieten een groeikoon aan het eind van de uitloper. Tevens zijn over het celoppervlak verspreid vingerachtige uitsteeksels te vinden. Tijdens de ontwikkeling speelt de groeikoon een essentiële rol bij het zoeken naar signalen die de groeirichting van de uitloper aangeven. In een later stadium zijn zowel groeikoon als vingerachtige uitsteeksels betrokken bij het vinden van een partner zenuwcel. Zowel de groeikoon als de vingerachtige uitsteeksels zitten vol met het motoreiwit actin, wat ze de voor dit proces noodzakelijke bewegelijkheid geeft. Gedurende de ontwikkeling worden deze structuren langzamerhand vervangen door structuren die we in de volwassen hersenen aantreffen. Axonen vormen stabiele verdikkingen, zogenaamde “varicosities” en dendrieten vormen stekelachtige of paddestoelachtige structuren die gelijkmatig over zijn oppervlak zijn verspreid, de zogenaamde “spines”. De verbindingen die deze nieuw gevormde structuren met elkaar aangaan vormen een zogenaamde synaps. Tezamen vormen zij de fundamentele structuur waarmee zenuwcellen met elkaar communiceren.

Om de ontwikkeling van zenuwcellen in de hersenen na te bootsen en te kunnen volgen heb ik zogenaamde orgaantypische kweken gemaakt van hersenweefsel. Het weefsel daarvoor is afkomstig uit transgene muizen die een fluorescente marker in hun celmembraan of een fluorescent gelabeld cytoskelet bevatten. Door de nog levende orgaantypische kweken met fluorescentie microscopie te bestuderen ben ik erin geslaagd de structuur van ontwikkelende zenuwcellen erg gedetailleerd vast te leggen. Door fluorescent gelabeld en niet-fluorescent gelabeld hersenweefsel tegelijkertijd te kweken in zogenaamde dubbelkweken, kon ik de ontwikkeling van zowel dendrieten in fluorescente weefsel als de invasie van fluorescent gelabelde axonen in niet-fluorescente weefsel volgen. Deze strategie bleek een daadkrachtige techniek te zijn om de vorming van neuronale netwerken te volgen.

In dubbelkweken van embryonaal weefsel vormden zenuwcellen langdurige wederzijdse interacties via hun axonale uitlopers. In de loop der tijd had zich een dicht neuronaal netwerk gevormd zichtbaar door de vele varicosities verspreid over de gehele lengte van het fluorescente axon. Dit in kweek gevormde neuronale netwerk bleef ten minste twee maanden lang in stand. Op de dendrieten ontwikkelden de labiele vingerachtige uitsteeksels, die in een vroeg stadium van de ontwikkeling werden geproduceerd, zich langzamerhand tot stabiele spines. De bewegelijkheid op het celoppervlak van zowel axonen als dendrieten verschoof daarmee geleidelijk aan richting synaps. Deze spines leken qua vorm sterk op degenen die eerder zijn beschreven in volwassen hersenen en ondergingen morfologische veranderingen die een behoud van plasticiteit in volwassen neuronale netwerken suggereren. De spontane en subtiele vormveranderingen van varicosities en spines suggereren dat ook zenuwcellen in volwassen hersenen nog steeds in staat zijn tot het verbeteren van verbindingen. Dit gebeurt bijvoorbeeld tijdens leerprocessen of ten gevolge van hersenbeschadigingen, waarbij verbindingen tussen zenuwcellen zich kunnen wijzigen.

Dubbelkweken van postnataal hersenweefsel lieten in de eerste twee weken in kweek een massale invasie van

axonale projecties zien. Daarna volgde een periode van dramatische axonale regressie die uiteindelijk leidde tot de totale afwezigheid van wederzijdse interacties tussen beide kweken. De ontwikkeling van dendrieten in beide postnatale kweken verliep in principe normaal en parallel met de ontwikkeling van dendrieten in embryonale kweken.

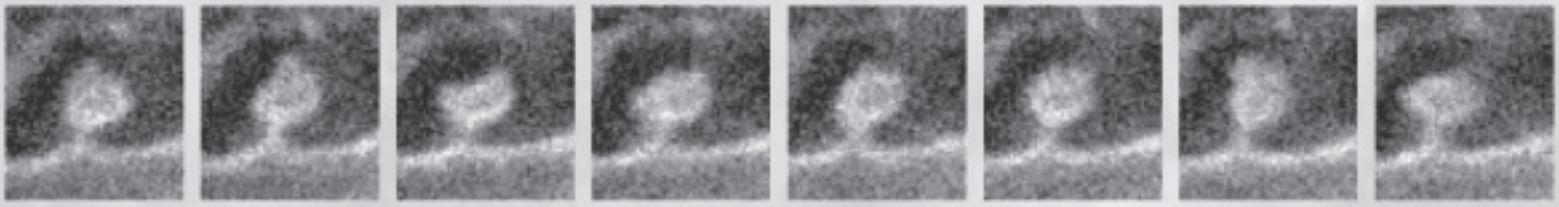
Om de oorzaak te ontdekken van het fundamentele verschil tussen postnatale en embryonale dubbelkweken, heb ik zogenaamde heterochrone dubbelkweken gemaakt. In deze dubbelkweken van postnataal en embryonaal hersenweefsel degenereerde de postnatale kweek waardoor er na een maand in kweek haast niets over was van het postnatale weefsel. De embryonale kweek die ernaast lag ontwikkelde zich normaal en zonder zichtbare problemen. Celspecifieke kleuringen van deze heterochrone dubbelkweken toonden aan dat microglia, de macrofagen van de hersenen die de overblijfselen van dode cellen opruimen, massaal de stervende postnatale kweek hadden omringd. Het fundamentele verschil tussen embryonale en postnatale dubbelkweken om de axonale interacties tussen beide kweken respectievelijk wel en niet in stand te houden suggereert een veranderingen in de moleculaire samenstelling van zenuwcellen. Deze veranderingen in de moleculaire samenstelling resulteren in heterochrone dubbelkweken voor een miscommunicatie tussen postnatale en embryonale cellen, wat leidt tot de verstrekkende degeneratie van postnataal weefsel. De dikke laag microglia die het postnataal weefsel omringt, suggereert de betrokkenheid van deze cellen bij de gevonden degeneratie van de zenuwcellen. Deze situatie is vergelijkbaar met de dood van zenuwcellen na de beschadiging van zenuwbanen of tijdens neurodegenerative condities zoals de ziekte van Alzheimer.

Door het afkoelen van volwassen neuronale netwerken werd de eerder gesuggereerde morfologische plasticiteit van synapsen bewezen. Spines zijn erg gevoelig voor verlaagde temperaturen en verliezen op kamertemperatuur al snel hun karakteristieke dynamische vormveranderingen. Binnen twaalf uur na het verlagen van de temperatuur was de hele spine structuur verdwenen van het oppervlak van de dendriet. Het opwarmen van deze gekoelde kweken naar lichaamstemperatuur leidde tot de vernieuwde vorming van dynamische vingerachtige uitlopers op het oppervlak van dendrieten en de hervorming van spines. Deze data benadrukken de hoge mate van plasticiteit die is behouden in neuronale netwerken in de volwassen hersenen en suggereren een samenhang tussen het gezonde functioneren van de hersenen en de aanwezigheid van spines.

ABBREVIATIONS

ACSF	Artificial cerebrospinal fluid
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid
BDNF	Brain derived neurotrophic factor
BoTX	Botulinum toxin
BSA	Bovine serum albumin
CaMKII	Ca2+/Calmodulin-dependent protein kinase II
CCB	Cacodylate buffer
CMV	Cytomegalovirus
dGBSS	Dissection Gey's balanced salts solution
DIV	Days in vitro
E18	Embryonic day 18
EPSP	Excitatory postsynaptic potential
GAD	Glutamic acid decarboxylase
GFP	Green fluorescent protein
GFPtKras	pβ-actin-EGFP-last20aa K-ras4B
HBSS	Hanks balanced salts solution
LDCV	Large dense core vesicle
LTP	Long term potentiation
MAP2	Microtubule associated protein 2
Munc13-1/2	Mammalian uncoordinated 13-1/2
Munc18-1	Mammalian uncoordinated 18-1
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
ON	Overnight
P8	Postnatal day 8
PFA	Paraformaldehyde
PSD	Postsynaptic density
SER	Smooth endoplasmatic reticulum
TTX	Tetrodotoxin
TUNEL	Terminal dUTP nick end labelling
VGCC	Voltage Gated Calcium Channel

INTRODUCTION



The brain is beyond doubt still one of the most fascinating structures in animals with many questions with regard to its functions still unresolved. Among the wide variety of cells known to mankind, neurons, as principle cells of the brain, possess probably the most complex morphology and behaviour both during and after development. It is beyond the scope of this thesis to deal with all details of neurogenesis, as it will take more than one's lifetime to describe. Instead I will start from the moment that principle cells in the brain are present at their proper location and describe from there on how the central nervous system (CNS) is wired together and how connections between the different neurons, synapses, are made. I will shortly introduce the brain structure that I have studied most, the hippocampus, and its principle cell, the pyramidal cell before moving on to the key players, dendritic spines.

Together they form some of the bits and pieces of the neuronal hardware. We can use it as we choose in various procedures, one of them being learning and memory, the neuronal software. As with all hard- and software, it is sensitive to various influences from the external and internal world, bugs, and I will describe some of them. This all together will hopefully give you enough background to understand the rationale behind my work.

The hardware

Neuronal network formation

From early in development on, neurons are polarized cells that have an asymmetric organization with two types of processes extending from the cell body. Both types of processes, axons and dendrites, are functionally and morphologically distinct. In vitro, neurons initially form various indifferent processes, neurites, each bearing a growth cone at its tip. One of these neurites elongates rapidly and become the axon; the other neurites will develop into dendrites (Dotti et al., 1988). Up to now it is unknown what the precise intracellular mechanism is that determines neurite fate. However, it is known that an increase in the actin dynamics and a local instability of the underlying actin meshwork in one of the growth cones are important determents in specifying neuronal polarization (Bradke & Dotti, 1999; Bradke & Dotti, 2000). Axon travel long distances and are guided along specific pathways to their target by an ingenious mechanism of attractive and repulsive cues (Mueller, 1999; Song & Poo, 2001; Dickson, 2002). Dendrites do not extend as far as axons from the cell body but instead branch extensively and give rise to a dendritic tree. Also dendrites are guided by numerous factors, each of them regulating the different decision points along the path, whether to branch, turn or stop (Cline, 2001; Scott & Luo, 2001; Whitford et al., 2002).

Both axonal and dendritic outgrowth, as many dynamic processes in nature, depend on actin dynamics (Luo, 2002). Small GTPases, key regulators of the actin cytoskeleton, have been shown to be essential for both axonal (Yuan et al., 2003) and dendritic (Wong & Wong, 2000; Scott et al., 2003) development. Finally, to limit growth of neuronal arbours at their target position Ca2+/Calmodulin-dependent protein kinase II (CaMKII) has been thought to be essential (Zou & Cline, 1999). Since not all is known about the precise mechanism behind the establishment of neuronal connectivity, many scientists have maintained in vitro slices of different parts of the brain in order to study path-finding (e.g. Gahwiler, 1981; Bolz et al., 1990; Molnar & Blakemore, 1991; Plenz & Kitai, 1996; Baker & van Pelt, 1997). Using this approach it was shown that axonal path finding is an intrinsically determined cascade of events (Molnar & Blakemore, 1999) that once completed, becomes fixed resulting in an inability of ingrowing axons in cortical regions to form synapses (Woodhams et al., 1993; Stoppini et al., 1997).

Synapse formation

Once dendrites and axons have found their appropriate target regions, synapses can be formed. Historically, data on synapse formation was acquired using immunohistochemical analysis of brain sections and electron micrographs. This static image of brain synaptogenesis suggested that the formation of synapses would take several days. Recent technical advances have made it possible to follow synaptogenesis live using low-density dissociated cell cultures of e.g. hippocampus (Banker & Goslin, 1998). These results suggested that individual glutamatergic synapses could be assembled within several hours (e.g. Ahmari et al., 2000; Friedman et al., 2000). During development synapses are formed in abundance i.e. more synapses are formed then eliminated (Marrs et al., 2001). This makes synaptogenesis a very dynamic process in which synapse formation and elimination go hand in hand (Jontes & Smith, 2000; Cohen-Cory, 2002). Both axons and dendrites extend filopodia from

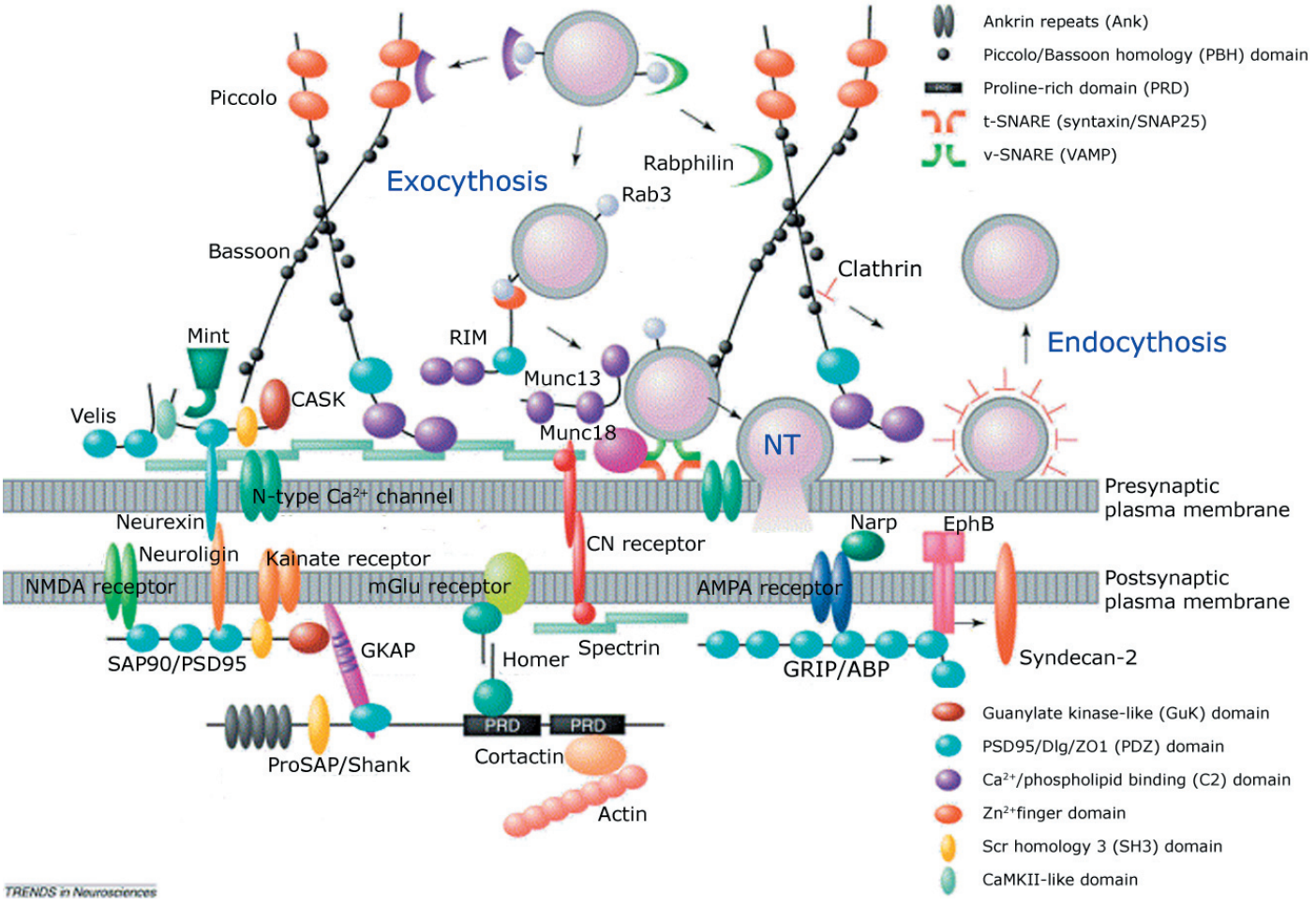


Figure 1: Pre- and postsynaptic machinery illustrating the vast complexity of the protein classes, including glutamatergic receptors (AMPA, NMDA, mGluR), scaffolding (PSD95, Shank, GRIP, Piccolo, Bassoon) and adapter molecules (GKAP, Homer, Mint, Synapsin), cell adhesion molecules (Neurexin, Neuroligin, Cadherin-related Neuronal receptor), proteins involved in synaptic vesicle recycling (SNAP-25, VAMP, Munc18), protein kinases/phosphataes (EphB, CASK) and the actin cytoskeleton, involved in the active zone where synaptic vesicles are released and the postsynaptic reception apparatus (From Garner et al., 2002). Together these proteins form a synapse.

their plasma membrane and sample the extracellular environment using both their growth cones and highly dynamic and actin-rich filopodia (Dailey & Smith, 1996; Wong & Wong, 2000; Chang & De Camilli, 2001; Roelandse et al., 2003) for potential pre- or postsynaptic partners (Jontes & Smith, 2000) in a time-dependent manner (Fletcher et al., 1994). The precise molecular mechanisms behind this process remain to be resolved however several cell-adhesion molecules have been suggested to be potential candidates for synapse initiation, including cadherins, neurexin/neuroligins and EphrinB/EphB receptors (Figure 1, Cohen-Cory, 2002; Garner et al., 2002). Once the initial contact has been established, most of the presynaptic machinery is delivered in pre-assembled macromolecular vesicular-associated complexes (Ahmari et al., 2000; Friedman et al., 2000; Shapira et al., 2003). These contain most of the synaptic vesicle proteins that together mature into functional synaptic vesicles (Südhof, 1995; Rizo & Südhof, 2002). Parts of these presynaptic differentiations precede postsynaptic differentiation, which might be explained by the fact that the active zone precursor vesicles are highly motile and distributed throughout the axon (Shapira et al., 2003). In contrast, postsynaptic filopodia are devoid of major components of postsynaptic reception apparatus (Figure 1), including scaffolding proteins like PSD-95 (Marrs et al., 2001; Okabe et al., 2001) and glutamate receptors (Washbourne et al., 2002a), and still lack the typical electron-dense structures at their tip (Fiala et al., 1998). Soon after the appearance of the active zone precursor vesicles, postsynaptic filopodia develop into protospines and spines and packets of scaffolding proteins and N-methyl-D-aspartate (NMDA)-type glutamate receptors move in (Prange & Murphy, 1999; Marrs et al., 2001).

The presynaptic plasma membrane forms a specialized meshwork of various proteins where synaptic vesicles

dock and fuse, the active zone (Figure 1, Zamorano & Garner, 2001; Martin, 2002). The postsynaptic machinery contains a thick electron-dense structure, the postsynaptic density (PSD) that accommodates a wide variety of protein classes including ionotropic receptors, scaffolding and adapter molecules, cell adhesion molecules, protein kinases/phosphatases and other signalling molecules. The PSD is tightly connected to the underlying actin cytoskeleton (Figure 1, Scannevin & Huganir, 2000; Yamauchi, 2002). The different glutamate receptor classes are transported to the postsynaptic membrane independently. NMDA receptors are rapidly transported along microtubules using transport packets that also contain specific scaffolding proteins as PSD-95 (Washbourne et al., 2002a) and arrive at the newly formed synaptic site within minutes after the establishment of the axodendritic contact. α-Amino-3-hydroxy-5-methyl-4-isoxazole-proprionic acid (AMPA)-type glutamate receptors are recruited to the synapse at a later stage and hence most synapses lack functional AMPA receptors early in development (Liao et al., 1999; Liao et al., 2001; but see Groc et al., 2002).

Dendritic spines

During postnatal development filopodia and protospines are gradually converted into dendritic spines, thereby increasing spine density over time, both in vivo and in vitro (Harris et al., 1992; Boyer et al., 1998; Kirov et al., 1999; Roelandse et al., 2003). Dendritic spines, as postsynaptic elements of mature excitatory synapses (Gray, 1959) differ in basic shape (Peters & Kaiserman-Abramof, 1970; Spacek & Hartmann, 1983; Harris et al., 1992; Trommald & Hulleberg, 1997), size and form of junctional zone (Peters & Kaiserman-Abramof, 1969; Spacek, 1985; Calverley & Jones, 1990; Geinisman, 1993) and distribution of different glutamate receptor subtypes relative to the synaptic junction (Somogyi et al., 1998; Takumi et al., 1999; Nusser, 2000). Using fixed and Golgi stained tissue or electron micrographs, spines have conventionally been divided into three distinct morphological categories, stubby (s), mushroom (m) and thin (t). These categories were based on differences in volume and length of neck and head (Figure 2, Peters & Kaiserman-Abramof, 1970). The PSD is located

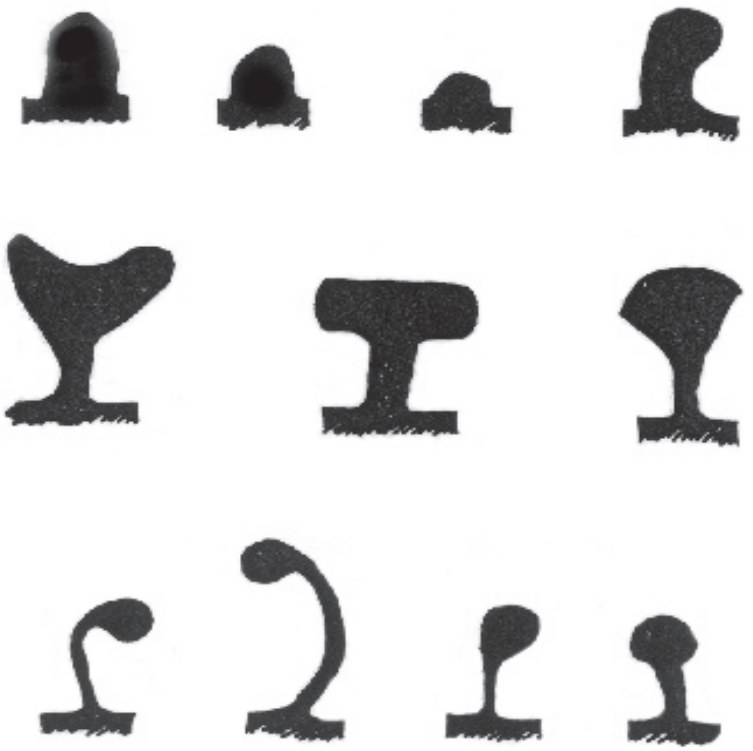


Figure 2: The conventional view of dendritic spines based on Golgi stained tissue and electron microscopy
Dendritic spines were, based on differences in total length of the spine and diameter of head and neck, classified in three distinct morphological categories: stubby (a), mushroom (b) and thin (c) (From Peters & Kaiserman-Abramof, 1970b)

on top of the spine head and contains both ionotropic AMPA- and NMDA-type glutamate receptors and metabotropic glutamate receptors; transmembrane proteins linked to a G-protein coupled second messenger system. Receptors and other proteins in the PSD are loosely connected with the actin-based cytoskeleton via several scaffolding and linker proteins (Adam & Matus, 1996; Bolton et al., 2000). The spine apparatus, a smooth endoplasmatic reticulum (SER)-like structure continuous with dendritic SER (Spacek & Harris, 1997), is located underneath the PSD. It also is thought to be connected to the actin cytoskeleton via linker proteins (Deller et al., 2000) and considered a donor of specific postsynaptic proteins as well as a major calcium store (Spacek, 1985; Berridge, 1998). The distinctive architecture of dendritic spines mainly depends on the underlying cytoskeleton.

In contrast to the dendritic shaft, whose cytoplasm is dominated by relatively stable microtubules (Kaech et al., 2001), dendritic spines are highly enriched with exclusively cytoplasmic isoforms of actin, α and β, which are targeted to spine heads (Matus et al., 1982; Kaech et al., 1997). This actin-based cytoskeleton forms longitudinal bundles in core regions of the head and neck and a fine meshwork of highly motile fibres at its periphery (Pavlik & Moshkov, 1991; Fischer et al., 1998). The latter finding suggests that the differences between spine types as observed in fixed tissue (Trommald & Hulleberg, 1997) may solely be based on snapshots of its rapid and spontaneous motility, which is typical of actin-rich structures. The actin cytoskeleton is also involved in the organization of dendritic glutamate receptors (Rosenmund & Westbrook, 1993; Allison et al., 1998; Hirai, 2000). These receptors are linked to molecules of the postsynaptic density (PSD) through multivalent adapter proteins (Matus, 1999; Garner et al., 2000; Scannevin & Huganir, 2000; Sheng & Pak, 2000).

The importance of the actin dynamics were underlined by the fact that dynamic actin filaments are essential for the long-term strengthening of synaptic connections (Kim & Lisman, 1999; Krucker et al., 2000). This suggests that dynamic actin filaments to participate in specific aspects of synaptic plasticity. The role of these dynamics in vivo appears to be most prominent during periods of sensory refinement and experience-dependent plasticity, the so-called critical period (Lendvai et al., 2000). Sensory deprivation by means of whisker trimming reduced the level of protrusive motility of dendritic spine suggesting that sensory activity can influence stability of dendritic protrusions and motility may thereby have a part in fine-tuning patterns of connectivity during brain development. The effect was prominent only during the critical period, 11 to 13 days after birth, which matches with the time span when animals start using their whiskers for exploratory activity, but was far less pronounced either before or after this period (Lendvai et al., 2000). There is thus a strong correlation between enhanced morphological plasticity in spines and experience-dependent refinement of circuit connectivity (Hering & Sheng, 2001), which is further supported by the decline in the incidence of spine motility during postnatal maturation (Dunaevsky et al., 1999).

Actin cytoskeleton

Actin molecules are single 375 amino acids long polypeptides that form 8 nm thick filaments consisting of a tight helix of uniformly oriented actin molecules. The filaments are polar structures with one slow growing end (minus) and another a fast growing end (plus). Addition of new actin molecules to an actin filament (polymerization) requires both adenosine 5'-triphosphate (ATP) and mono- and divalent cations (e.g. K^+ and Mg^{2+}). After polymerization, the actin-bound ATP is hydrolyzed and the resulting adenosine 5'-triphosphate (ADP) is trapped in the polymer. The hydrolysis is essential for the dynamic behaviour of actin filaments (Carlier & Pantaloni, 1997). The addition and removal of actin molecules to existing filaments is a dynamic equilibrium

between the filaments and actin molecules (Alberts et al., 1994). During development filamentous actin is transported to pre- and postsynaptic sites in an activity dependent manner (Colicos et al., 2001) and becomes transiently concentrated in excitatory postsynaptic compartments (Zhang & Benson, 2002). Filamentous actin is enriched in mature dendritic spines (Matus et al., 1982; Morales & Fifkova, 1989) that lack other cytoskeletal elements as microtubules or intermediate filaments (Kaech et al., 2001). During development actin dynamics are essential for synapse formation but actin filaments become over time more and more stable and resistant to depolymerisation (Zhang & Benson, 2001) suggesting a correlation between actin stability and a focalization of morphological plasticity (Luo, 2002). The actin cytoskeleton in spines is essential for the rapid shape changes since drugs that inhibited actin polymerization such as Latrunculin B and Cytochalasin D can block their morphological plasticity (Dailey & Smith, 1996; Ziv & Smith, 1996; Fischer et al., 1998; Dunaevsky et al., 1999; Lendvai et al., 2000).

Spines are also enriched with proteins that regulate actin polymerization and control the arrangement of actin filaments, thereby regulating spine shape and motility (e.g. drebrin (Hayashi & Shirao, 1999) and CaMKII (Shen et al., 1998)). Upon Ca2+ influx through the NMDA receptor channel, actin filaments depolymerise, resulting in a selective loss of actin filaments in dendritic spines (Halpain et al., 1998), and a decreased number of AMPA and NMDA receptor clusters (Allison et al., 1998). Therefore, synaptic transmission can potentiate depolymerisation of actin filaments in spines and thereby regulate spine shape and motility. Important proteins that link the actin cytoskeleton to the PSD and its glutamate receptors are e.g. α-actinin-2 (Wyszynski et al., 1997) and synapse associated protein (SAP) 97 (Müller et al., 1995). α-Actinin-2 is specifically concentrated in developing spines but is absent in shaft synapses (Rao et al., 1998) and links actin filaments to NMDA receptors. However, this cross-linkage between actin and NMDA receptors can be dissociated upon Ca2+ influx (Zhang et al., 1998; Krupp et al., 1999), thereby disrupting one of the associations of the actin cytoskeleton to the plasma membrane. The expression of SAP97 is not restricted to the CNS and is thought to play a role in cell adhesion (Muller et al., 1995). SAP97 links AMPA receptors to the actin cytoskeleton (Leonard et al., 1998), suggesting that it may be involved in the clustering of AMPA receptors at the postsynaptic sides.

Glutamate receptors

As mentioned before, glutamate receptors can be subdivided in two major classes, ionotropic (NMDA receptors, composed of subunits NR1, NR2A-2D; AMPA receptors, composed of subunits GluR1-4; and kainate receptors, composed of subunits GluR5-7) and metabotropic glutamate receptors (composed of subunits mGluR1-7) (Andersen et al., 1966; Hollmann & Heinemann, 1994). The expression of the different excitatory receptors and their properties largely depends on the source of innervation (Gottmann et al., 1997). Early in development most excitatory synapses contain only NMDA receptors and are called 'silent synapses' (Malenka & Nicoll, 1997). The binding of glutamate to NMDA receptors may result in the opening of the receptor channels but not in a postsynaptic potential since the ion channel pore is still blocked by Mg2+. Depolarization of these silent synapses results in a subsequent recruitment of AMPA receptors to the membrane (Liao et al., 2001).

During development, receptors gradually change their responsiveness to neurotransmitter (Wong et al., 2000; Ben-Ari, 2002) as do the expression patterns. The number of AMPA receptors slowly increases, whereas the number of NMDA receptors, that are already present at the synapse in large amounts, remains stable, suggesting a decreasing number of silent synapses over time (Petralia et al., 1999). The latter finding has recently become a

matter of debate since others have found similar numbers of AMPA receptors as compared to NMDA receptors already during early postnatal development (Groc et al., 2002). In area CA1 of the hippocampus, within the postsynaptic density, AMPA and NMDA receptors are differentially distributed across the synaptic surface; AMPA receptors are evenly distributed whereas NMDA receptors are predominantly localized in the centre of the PSD (Somogyi et al., 1998). GluR1 and NR1 subunits co-localize in 25% of mature dendritic spines, 45% of the spines are NR1 positive and 30% is GluR2 positive (He et al., 1998). The presence of AMPA receptors on synapses is also determined by the diameter of the PSD; PSDs smaller than 180 nm only have NMDA receptors (Takumi et al., 1999). Consequently, AMPA receptors are abundant on the large-headed mushroom and stubby spines and nearly absent on thin spines and filopodia (Matsuzaki et al., 2001). In addition, perforated synapses, with a split or irregular PSD, are twice as likely to express AMPA receptors as non-perforated ones whereas no difference has been noted for NMDA receptors (Desmond & Weinberg, 1998). Dendrites can form new spines in response to activation of NMDA receptors (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999) while existing spines may degenerate when NMDA receptors are hyper-stimulated (Choi, 1994; Halpain et al., 1998). AMPA receptors also influence spine structure, stabilizing spine morphology when they are activated (Fischer et al., 2000) and leading to their regression when they are blocked (McKinney et al., 1999b). In an attempt to further unravel the role of glutamate receptors in learning and memory, AMPA receptor antagonists have been infused in the hippocampus of an awaken animal during or after performing a memory task. This revealed that AMPA receptors are especially needed for the encoding and retrieval of spatial memory (Riedel et al., 1999).

Together it is likely that shape and location of dendritic spines play a major role in determining patterns of signal flow in the CNS. Both during development and in adult CNS, dendrites are morphologically plastic, showing changes depending on experience and activity, which can involve alterations in both length and branching of dendrites and number of dendritic spines.

The hippocampus

The hippocampal formation is located in the forebrain (prosencephalon) and consists in the adult animal of several distinct areas: Cornu Ammonus-1 (CA1), CA2, CA3, Dentate Gyrus and Entorhinal Cortex (Figure 3). In the hippocampus, information flows from entorhinal cortex (ento) to dentate gyrus (red) via the perforant pathway (pp). Dentate granule cells relay information to area CA3 via mossy fibre pathway (mf). CA3

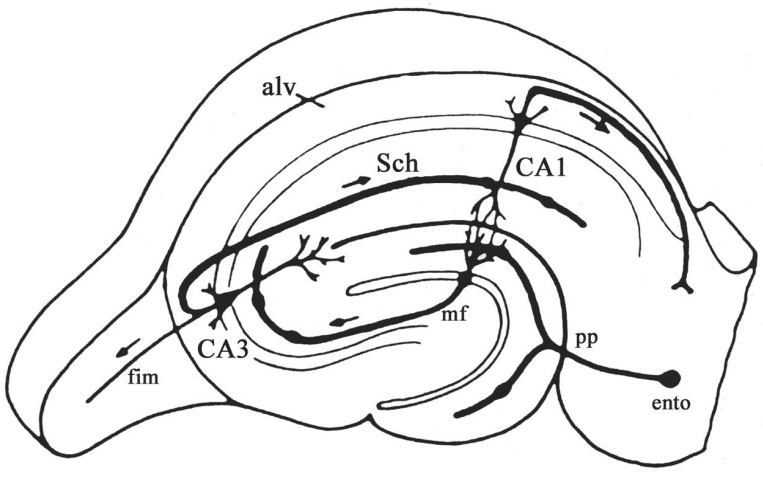


Figure 3: Location of the different regions of the hippocampus
CA1, CA3, dentate gyrus (DG) and entorhinal cortex (ento), and the three major excitatory pathways of information flow within the hippocampus, perforant pathway (pp), mossy fiber pathway (mf) and Schaffer collaterals (Sch) (See text for details, from Shepherd, 1994).

pyramidal axons project through Schaffer collaterals (Sch) to area CA1 or via the fimbria (fim) to subcortical regions and via the commissural/associational pathway to either contra lateral pyramidal cells (commissural), or to neighbouring pyramidal cells (associational). CA1 pyramidal cells project via the subiculum to subcortical areas (Shepherd, 1994). Major inputs to the hippocampus are olfactory bulb, amygdala, associated cortex, claustrum, medial septum, thalamus and midbrain (Seifert, 1983). Cellular organization in area CA1 and CA3 is very similar. Alveus (alv) and stratum oriens form the outer layer and contain basal dendrites that originate in the pyramidal cell layer (blue). Primary apical dendrites form the stratum lucidum, whereas the secondary apical dendrites occupy a wide band of the hippocampus, called the stratum radiatum. The dendrites then end in the stratum lacunosum, or molecular layer (Ishizuka et al., 1995). The principal cells of the hippocampus, pyramidal neurons, are introduced underneath.

Pyramidal neurons

Pyramidal cells are excitatory neurons that use glutamate as a neurotransmitter. Their dendritic organization facilitates the integration of a variety of inputs. Pyramidal neurons in the different regions of the hippocampus differ in detail. CA1 pyramidal cells are smaller than CA3 pyramidal cells; their mean cell body area is 190 μm2 whereas that of CA3 pyramidal cells is 460 μm2. CA2 pyramidal cells are a mixture of CA3- and CA1-like cells but lack dendritic thorny excrescences on their apical and basal dendrites. CA1 and CA3 pyramidal cells also differ in location of dendritic length (Ishizuka et al., 1995), which inevitably may have an influence on the input to the pyramidal cells. In addition, the total length of axon collaterals and density of the axonal network of pyramidal cells in CA1 is more extensive then in the CA3 (Li et al., 1994). The axons of the CA3 pyramidal cells often run deep into the CA1 molecular layer. There, they project onto dendritic spines. On average, one CA3 pyramidal cell has 20.000 synapses with CA1 pyramidal cells (Andersen, 1990). Here, information is transferred from the presynaptic bouton to the postsynaptic spine.

The software

The previous paragraphs have given a short outline of the structural elements of the brain. Using this hardware we can manage all the various belongings of our body as well as adapt to new situations and communicate with the outside world. The following paragraphs describe one of the plastic processes that take place in the brain, learning and memory.

Learning and memory

In the past, learning and memory have been studied intensively using animal behaviour. This resulted in the distinction between memory types based on observations. One type of memory is declarative memory, which depends on conscious attention and recall of facts, such as an individual's surrounding (spatial memory), orientation in time (temporal memory), or the account of recent past (episodic memory). Declarative memory relies on cognitive processes such as comparison, evaluation, and inference. It involves the processing of bits and pieces of information that the brain can use to reconstruct past events or episodes. Which precise brain areas are involved in the processing of declarative memory is still a matter of debate. Some interpreted the current data as such that declarative memory is depending on hippocampus function (Squire & Zola, 1996; Alkire et al., 1998), but others suggest a much wider selection of brain areas (Cabeza et al., 1997; Vargha-Khadem et al., 1997). Another type of memory is non-declarative memory which implies knowing how to perform a skilled act. It accumulates slowly through repetition over many trials and is expressed primarily in terms of improved performance on certain tasks. Non-declarative memory is thought to involve striatum, motor cortex, cerebellum, and entorhinal cortex (Zola-Morgan & Squire, 1993). However, constant repetition of a task can change non-declarative memory into reflexive memory, so that a task which originally required conscious attention now becomes an unconscious performed motor routine, for example the acquisition of balance required to ride a bike.

Brain events leading to memory

Several concepts of the mechanisms leading to the process of learning and memory are generally accepted. One hypothesis is that 'saving' memory occurs in several stages. The initial stage is when information is encoded in neuronal circuits. The next step, known as consolidation, is the transformation of information to its long-term storage place. Finally, from there, it should be able to be retrieved for usage as perceptual or motor output (Shepherd, 1994). Some studies suggest a reorganization of neural circuitry as underlying mechanism for long-term storage (Bontempi et al., 1999). The retrieval of recent memory can be disturbed by accidents or short-term cardiovascular malfunction however only up until memories have been converted to long-term memory. Over time stored information and the capacity to retrieve it are gradually lost. Another widely accepted idea is that storage of long-term memory may be embedded in plastic changes in the brain at the protein level, i.e. proteins involved in synaptic transmission have changed functional properties. The plastic changes encoding memory are often localized in different places throughout the nervous system. Many if not all regions in the brain appear to contain neurons with properties of plasticity needed for memory storage. However, the exact memory traces in the brain (engram) are still unknown.

Molecular mechanisms of declarative memory

In order to unravel molecular mechanisms of declarative memory past research has been focused on the role of hippocampus in spatial memory. It was discovered that high frequency stimulation of a bundle of axons results in a long-lasting increase in the excitatory postsynaptic potential (EPSP); this was called long-term potentiation (LTP) (Bliss & Lomo, 1973). This potentiation could be induced in several pathways, e.g. the Schaffer collateral. One train of high-frequency stimulation on CA3 axons results in a short-lasting change in electric response of CA1 synapses. Four or more trains of stimuli induce a changed electric response that lasts up to 24h. To induce LTP in synapses, a sufficient number of inputs, that is, at least more than one fibre must be activated (cooperatively). For a weak stimulus to become potentiated, contributing fibres and the postsynaptic cell need to be activated together (associatively). LTP is specific to the stimulated pathway; it will not be elicited in other unstimulated pathways (specificity).

The spontaneous release of one presynaptic vesicle (quanta) with glutamate in the synaptic cleft results in the binding of glutamate to both AMPA and NMDA receptors and a subsequent short-lasting change in the membrane potential (mEPSP). Na^+- and K^+-ions will flow through AMPA receptor channels but Mg^{2+}-ions will still block NMDA receptor channels. When more glutamate is released from the presynaptic terminal, the change in membrane potential will be large enough to relieve the Mg^{2+}-ion from its NMDA receptor and Na^+-, K^+-, and Ca^{2+}-ions will flow through the NMDA-receptor channel. This results in a maximum depolarization of the membrane (Bliss & Collingridge, 1993) and the opening of voltage-gated Ca^{2+} channels (VGCC). The Ca^{2+} concentration within the postsynapse increases which in turn will trigger a variety of secondary metabolic events including the activation of calcium-dependent kinases (Malinow et al., 1988; Malenka et al., 1989; Malinow et al., 1989; O'Dell et al., 1991; Grant et al., 1992). Afferent stimulation can also trigger a decrease in responsiveness of the postsynaptic cells, a process called long-term depression (LTD) and long being considered to be the reversal of LTP or a prospective physiologic event causing 'forgetting' (Bear, 1999). LTD can be induced in the Schaffer collateral pathway by delivering a train of electric stimuli at a low frequency, resulting a depression in electrical response of synapses that persists for several hours (Linden, 1994). Similar to LTP induction (Collingridge et al., 1983), NMDA receptor antagonists can reversibly block the induction of LTD, suggesting that postsynaptic Ca2+ influx is critical for LTD (Dudek & Bear, 1992). Once LTD has been induced in a certain synapse, it can be potentiated again, indicating that the depression is not due to a damaged synapse and LTD can thereby be reversed to LTP and vice versa (Mulkey & Malenka, 1992). It is unclear is how Ca^{2+} entry through the NMDA receptor channel can trigger both LTP and LTD. One possibility is that a modest elevation causes a selective activation of protein phosphatases, as LTD can be blocked by the addition of protein phosphatase inhibitors, while large increases in the intracellular Ca^{2+} activates a whole range of signalling cascades (Mulkey et al., 1993). The balance between activated kinases and phosphatases acting on the same substrate would thereby regulate synaptic effectiveness.

Bugs

As with many systems, things can go wrong. Also in the brain things can go skewed due to genetic alterations or outside influences. Some examples with a focus on dendritic spines and synapses are described in the following paragraphs to underline the plasticity of the brain.

Dendritic spines and mental retardation

Over the last decades a growing body of evidence has accumulated for a correlation between an altered dendrite morphology and mental retardation (Marin-Padilla, 1975; Kaufmann & Moser, 2000; Ramakers, 2000). These changes in morphology, as observed in post-mortem tissue from mentally retarded patients (Figure 4), involve very thin and long, tortuous spines with prominent heads, and a relative absence of normal and stubby spines. These morphological changes of spines are accompanied with decreased total spine density, both present in genetically inherited disorders such as Fragile-X syndrome (Hinton et al., 1991; Irwin et al., 2000) and Down's syndrome (Marin-Padilla, 1976; Suetsugu & Mehraein, 1980), as well as in induced syndromes as foetal alcohol syndrome (Stoltenburg-Didinger & Spohr, 1983) and phenylketonuria (Bauman & Kemper, 1982; Lacey & Terplan, 1987) or syndromes of unknown aetiology (Gonatas & Moss, 1975; Purpura, 1979; Dietzmann & von Bossanyi, 1994). These data strongly suggest that changes in spine morphology result in a distortion of mental fitness.

Synaptic connections and the influence of temperature

An extreme form of neuronal plasticity may well be the process of hibernation. This biological phenomenon, induced by low temperatures, results in a complete reorganization, from molecular to behavioural changes. Hibernating animals have body temperatures between 0˚C and 8˚C and spent most of the time in torpor with only short periods of arousal (Hut et al., 2002). Past studies have shown that despite the low body temperature and low metabolic and neuronal function (Derij & Shtark, 1985; Krilowicz et al., 1989; Igelmund & Heinemann, 1995), their brain is able to adapt to these annual periods of hibernation. During the entry to torpidity, electrical activity of brain structures is sequentially suppressed from cortex to brainstem, thalamus and finally hippocampus, and during awaking in reverse order (Derij & Shtark, 1985). Not only were these areas electrically silenced, the decrease brain temperature resulted in a rapid and profound transformation of all structural elements; dendrites were significantly shorter, less branched and had fewer dendritic spines in hippocampus and cerebellum during the course of hibernation (Boycott, 1982; Popov et al., 1992). The extreme plasticity of the system lies in the fact that these structural changes can be completely restored within 2h after arousal (Popov & Bocharova, 1992; Popov et al., 1992). In contrast, areas needed during the period of torpor, such as the supraoptic nucleus that is involved in the regulation of body temperature, selectively strengthen their connections. There, neurons have an increased degree of branching and an increased number of spines during the course of hibernation (Sanchez-Toscano et al., 1989). However, hibernating animals do pay a price for these adaptations to the cold. Comparing hibernating animals with non-hibernating littermates in an operant learning task and a spatial memory task showed that non-hibernating animals performed better in tests for retained memory such as the Morris water maze (Millesi et al., 2001), suggesting that some information was

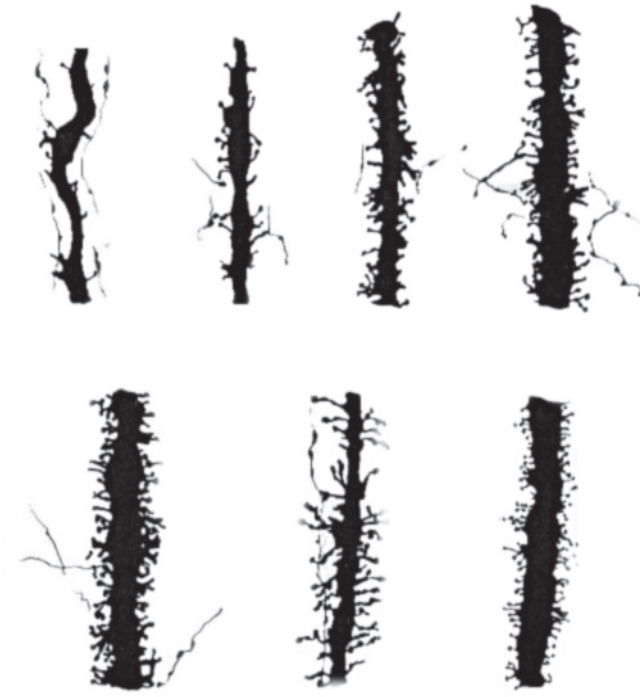


Figure 4: Gradual increase in the numbers of spines during pre- and postnatal development in contrast to decreased spine numbers and malformed mature spines in patients with mental retardation (From Purpura et al., 1979)

lost during the hibernation. As for the morphological changes, the effects of hibernation on memory retention were not universal; both hibernators and non-hibernators performed equally well in a social recognition task (Millesi et al., 2001).

Apart from animals like European ground squirrels or hedgehogs that are build to survive long lasting decreased body temperature, other animals also have some resistance against a low body temperature and can survive short periods of so-called hypothermia by adapting their ATP-balance of demand and supply (Boutilier, 2001) and by a subsequent burst-suppression and electro-cerebral silence in their brain (Stecker et al., 2001). Similarly, a strong correlation was found between brain temperature and state of consciousness (Mathew et al., 2001). Decelerating metabolic and neuronal function by means of hypothermia has been proven to be of advantage in neuronal survival and recovery after injury (Yu et al., 2000), e.g. to protect brain function during cardiovascular operations with a temporary absence of cerebral perfusion (Coselli et al., 1988; Mizrahi et al., 1989; Moshkovitz et al., 1998; Kouchoukos et al., 2001) or as 'cryo-anaesthesia' in neonatal animals (Phifer & Terry, 1986). Even though cryo-anaesthesia is widely used, it was shown that a short exposure of an animal in the critical period has an impact on several structural elements in the brain. A 30 min exposure of neonatal rat to 4˚C results in a decreased volume and numbers of neurons and glia in the adult visual cortex (Nunez et al., 1998) and hippocampus, as well as a poorer performance in a spatial memory task (Nunez et al., 2000). The fact that these effects only occur in neonatal animals and not mature animals (Kolb & Cioe, 2001) suggests that the temporary electro-cerebral silence alters late developing brain structures like hippocampus or cerebellum. This hypothesis is strengthened by the fact that neonatal exposure to volatile anaesthesia results in similar changes in volume and numbers of neurons and glia in the adult visual cortex (Nunez & Juraska, 2000). It should be noted that decreasing brain temperature also diminishes or eliminates the need for volatile anaesthesia (Vitez et al., 1974; Antognini, 1993; Liu et al., 2001), suggesting a strong correlation between the underlying mechanisms responsible for the electro-cerebral silence during hypothermia and due to volatile anaesthesia.
Various studies have attempted to unravel the underlying effectors of hypothermia by examining the differ-

ent neuronal parameters at different temperatures. Several publications have shown a substantial difference between several electrophysiological parameters at room temperature (24˚C) and physiological temperatures (37˚C) in a variety of brain slices and ages (Borst & Sakmann, 1998; Masino & Dunwiddie, 1999; Volgushev et al., 2000; Aihara et al., 2001). Nonetheless, the majority of electrophysiological experiments are still pursued at room temperature. Decreasing temperature results in an increased membrane potential in CA3 pyramidal cells (Aihara et al., 2001), layer 2/3 pyramidal cells of the visual cortex (Volgushev et al., 2000) as well as an increase in CA1 field potentials (Masino & Dunwiddie, 1999). In general, at physiologic temperatures connection between neurons are more reliable due to less trial-to-trial variability and fewer failures of synaptic transmission (Hardingham & Larkman, 1998; Pyott & Rosenmund, 2002). As compared to room temperature, at physiologic temperatures hippocampus neurons have an increase in the size and refilling rate of the ready release pool of neurotransmitter and an increase in both amplitude and frequency of spontaneous release of neurotransmitter (mEPSP). The change in amplitude can be explained by an increased opening probability or conductance of the postsynaptic receptor whereas the increased frequency of mEPSP's is caused by the decreased energy requirement for spontaneous fusion due to an increased fluidity of the lipid bi-layer at higher temperatures (Masino & Dunwiddie, 1999). Furthermore, the width and total area of action potentials decreases (Volgushev et al., 2000) despite an increase in peak amplitude (Borst & Sakmann, 1998). These results can be explained by an increased time course of neurotransmitter in the synaptic cleft at lower temperatures (Silver et al., 1996) together with higher opening probabilities of the Ca^{2+}-channels at higher temperature (Borst & Sakmann, 1998). Together these results suggest a decreased presynaptic release probability, a slower diffusion of the neurotransmitter through the synaptic cleft but a decreased desensitization of the postsynaptic receptor and an increased membrane potential at room temperature. Therefore with decreasing temperature, neurons become more and more hyperexcitable until a depolarization block at temperatures below 10 ˚C which will subsequently inactivate them. It should be noted however that the observed changes in electrophysiological properties after short periods of decreased temperatures recovered fully (Volgushev et al., 2000; Aihara et al., 2001).

Dendritic spines and the influence of synaptic transmission

In the past many groups questioned the influence of synaptic transmission on both neuronal networks and spine formation and maintenance (for review, see Jontes & Smith, 2000; Matus, 2000; Zhang & Poo, 2001; Ziv & Garner, 2001; Lopez-Bendito & Molnar, 2003). Many of these studies use drugs that block action potential-dependent (tetrodoxin, TTX) or action potential-independent (Botulinum toxin, BoTX) release of neurotransmitter from the presynaptic bouton, or a cocktail of drugs that block all glutamate receptor variants, and were applied to neuronal cultures (Kossel et al., 1997; Okabe et al., 1999), organotypic cultures (Baker & van Pelt, 1997; Drakew et al., 1999; Kirov & Harris, 1999; McKinney et al., 1999a) or injected in the brain of a living animal (Bravin et al., 1999). Whereas TTX mainly influences maturation of dendritic spines (Drakew et al., 1999; Kirov & Harris, 1999) and elongation of dendrites and axons (Baker & van Pelt, 1997; Bravin et al., 1999; Okabe et al., 1999), abolishing network activity using BoTX has more devastating effects. Spine densities were reduced to half the control values, an effect also seen after transection of the axonal pathway (McKinney et al., 1999a). Together this suggests a role for electrical activity in maintenance and fine-tuning of neuronal connections by branching and elongation of neurites and the growth of new spines.

Besides the usage of the different drugs described above, there are also animal models in which essential pre- or postsynaptic components of the synaptic transmission machinery have been eliminated. One of these compo-

nents is one of the odorant receptors, OCNC1, of which the gene is located on the X-chromosome. When this gene is inactivated, odorant receptor mediated activity is abolished. Nevertheless, axons converge properly to their target and dendrites are normally refined in male knockout mice (Lin et al., 2000). In hemizygote females, neurons lacking this receptor also develop normally and project their axons to proper targets in the glomeruli up to P7. The competitive environment will then subsequently eliminate knockout neurons in favour of wild-type cells (Zhao & Reed, 2001). This suggests that presynaptic activity is critical for neuronal survival. Additional evidence for this hypothesis is given in a study where one of the proteins involved in synaptic vesicle release, munc 18-1, is abolished. Although the gross brain assembly evolved normally in these animals, after assembly was completed neurons underwent apoptosis, leading to neurodegeneration (Verhage et al., 2000).

Aim

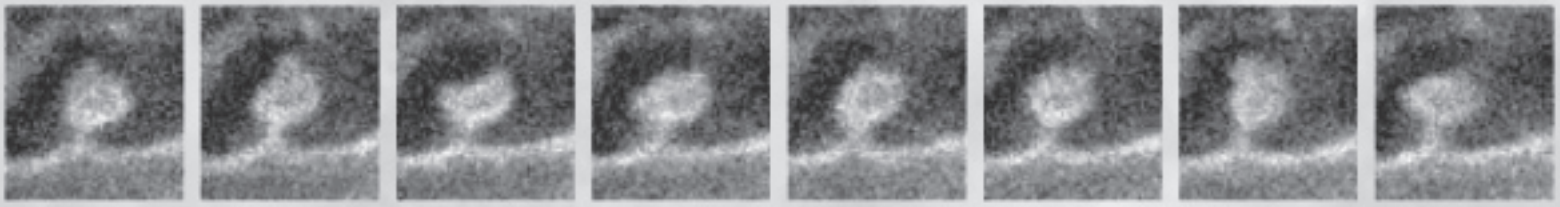
During my Ph.D.-project in the laboratory of Prof. Dr. Andrew Matus, I have studied the formation of neural networks thereby acquiring an impression of basic development of both pre- and postsynaptic specialisations, axonal bouton and dendritic spines. First, I studied organotypic cultures of eight day old transgenic mice (Gahwiler et al., 1998) and imaged dendritic spines in these cultures using our Yokogawa microlens Nipkow confocal live cell imaging system. I also analyzed the fine structure of brain tissue, and particularly dendritic spines, using electron microscopy. To further study formation of neural networks, I have set up cultures from embryonic day 18 (E18) or postnatal day 8 (P8) hippocampus and in order to achieve a suitable model for examining both pre- and postsynaptic structures at the same time use co-cultures of fluorescent and non-fluorescent slices that would allow me to studied the dynamics and behaviour of developing axons originating from GFP-expressing neurons as well as those of protrusions and spines on developing dendrites before and after network formation.

To study the role of synaptic transmission on determining synapse morphology and plasticity, I have established collaboration with Prof. Dr. M. Verhage (UMC Utrecht, NL) and used his Munc 18-1 knockout mice that lack synaptic vesicle release and hence synaptic transmission (Verhage et al., 2000). Crossbreeding heterozygote knockout Munc18-1 mice with transgenic mice expressing a cell surface marker construct, GFP-tKras will then provide me with double transgenic mice. Using single slices of fluorescently labelled knockout mice will enable me to answer several questions, as to whether we can maintain these cultures as it is of do they need the addition of certain (specific) neurotransmitter agonists or a depolarizing environment to prevent them from dying (see Lin et al., 2000; Verhage et al., 2000). Once I would be able to keep knockout neurons alive, other questions arise as to whether dendritic spines form in the absence of actively releasing presynaptic terminals (see Kossel et al., 1997), and if they whether they differ in the degree of morphological plasticity as compared to control cells (see Lendvai et al., 2000c). If the presence of a presynaptic terminal alone is not sufficient for a dendrite to develop spines, I will try to manipulate dendrites to grow spines by changing the culturing medium or by addition of agonists (see Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999).
I will further investigate the role of synaptic transmission on spine formation and maintenance by setting up organotypic co-cultures of hippocampus from mixed origin. By using one slice from Munc 18-1 knockout mice and one from wild-type in combination with either of them expressing different cell surface markers, I will be to study the behaviour of a actively releasing axon entering tissue containing ‘silenced’ axons and the response

of the surrounding dendrites and vice versa. An initial question will be whether these silenced axons do grow out in the first place (see Baker et al., 1997; Drakew et al., 1999) and if so, whether these 'silenced' axons will innervate existing spines or whether they will form new spines in wild type tissue. This will resolve the question whether spine formation is an intrinsic process of the dendrite itself or whether this is an activity dependent process (see Baker et al., 1997). Vice versa, will actively releasing axonal bouton growing in munc18 tissue form spines on existing dendrites, or will they compete with the present axons for the same spines, and what is the effect on the connecting neurons (see Zhao & Reed, 2001).

Together this would give us an idea as to how stable connections are in an existing neuronal network under changing synaptic activity and in addition enable me to answer some basic questions regarding the morphological stability of synaptic connections in the brain. This in turn should provide new insights into the plasticity of synaptic connections in the normal brain as well as in pathological conditions.

FOCAL MOTILITY DETERMINES THE GEOMETRY OF DENDRITIC SPINES



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Abstract

The geometry of dendritic spines has a major impact on signal transmission at excitatory synapses. To study it in detail we raised transgenic mice expressing an intrinsic green fluorescent protein (GFP)-based plasma membrane marker that directly visualizes the cell surface of living neurons throughout the brain. Confocal imaging of developing hippocampal slices showed that as dendrites mature they switch from producing labile filopodia and polymorphic spine precursors to dendritic spines with morphologies similar to those reported from studies of adult brain. In images of live dendrites these mature spines are fundamentally stable structures, but retain morphological plasticity in the form of actin-rich lamellipodia at the tips of spine heads. In live mature dendrites up to 50% of spines had cup-shaped heads with prominent terminal lamellipodia whose motility produced constant alterations in the detailed geometry of the synaptic contact zone. The partial enveloping of presynaptic terminals by these cup-shaped spines coupled with rapid, actin-driven changes in their shape, may operate to fine tune receptor distribution and neurotransmitter cross talk at excitatory synapses.

Key Words: synaptic plasticity, neuronal development, morphology, hippocampus, time-lapse microscopy, green fluorescent protein

Introduction

The geometry of dendritic spines is a major determinant of synaptic transmission at excitatory synapses, influencing the number and distribution of glutamate receptor subtypes (Rao et al., 1998; Takumi et al., 1999; Nusser, 2000; Racca et al., 2000; Matsuzaki et al., 2001) and significantly affecting the spread of postsynaptic Ca2+ fluxes from spine to dendrite (Muller & Connor, 1991; Denk et al., 1996; Eilers & Konnerth, 1997; Koester & Sakmann, 1998; Majewska et al., 2000). By determining extra-junctional path length, spine shape may also influence the spillover of neurotransmitter from one synaptic release site to activate receptors at another (Clements, 1996; Barbour & Häusser, 1997; Isaacson, 2000; Kullmann, 2000). Changes in spine morphology are also widely believed to be involved in the modulation of synaptic connectivity underlying learning and memory (reviewed by Halpain, 2000; Luscher et al., 2000; Matus, 2000; Segal & Andersen, 2000; Wong & Wong, 2000; Yuste & Bonhoeffer, 2001).

These diverse influences of dendritic spine morphology on synaptic function underline the importance of accurately determining spine shape and its variation, particularly since recent studies have shown that dendritic spines are enriched in dynamic actin filaments that produce rapid changes in their shape (Kaech et al., 1997; Fischer et al., 1998; Dunaevsky et al., 1999; Fischer et al., 2000; Dunaevsky et al., 2001; Kaech et al., 2001; Korkotian & Segal, 2001; Star et al., 2002). At early stages of development, immature dendrites produce motile filopodia and nascent "protospines" which undergo extensive changes in morphology (Dailey & Smith, 1996a; Jontes & Smith, 2000; Lendvai et al., 2000; Wong & Wong, 2000; Marrs et al., 2001). However, as development proceeds this extreme morphological plasticity is substantially down-regulated (Ziv & Smith, 1996; Dunaevsky et al., 1999; Lendvai et al., 2000) and the degree to which changes in the shapes of dendritic spines persist in the adult brain remains uncertain.

Many past studies have relied on injecting fluorescent dyes or transfecting soluble green fluorescent protein (GFP) into single neurons, circumstances, which make it difficult to control the amount dispensed or to eliminate possible non-specific effects of the marker molecule. Additionally, intense fluorescent signals from the spine interior produced by soluble space-filling markers make it difficult to resolve fine structural details of the dendritic spine surface. To address these problems we generated transgenic mice in which neurons throughout the brain stably express GFP-tKras, a plasma membrane marker that provides high resolution images of spine morphology based on signals emanating directly from the neuronal surface. By avoiding artefacts associated with microinjection or transfection, these animals provide a reproducible source of tissue for live cell imaging. Aside from expressing the fluorescent marker they do not differ observably from either wild-type mice non-transgenic littermates, indicating that their CNS tissue is functionally normal.

Results from time-lapse imaging of GFP-tKras labelled neurons combined with electron microscopy of tissue from wild-type mice show that as dendrites mature, morphological plasticity becomes restricted to motile actin-rich lamellipodia on dendritic spine heads which partially enclose presynaptic boutons, a configuration with potentially important implications for the distribution of postsynaptic receptors and the spread of neurotransmitter between synapses.

Methods

Transgenic animals

Transgenic animals expressed GFP-tagged constructs under control of a chicken β-actin promoter (Ludin et al., 1996). Actin-GFP transgenic mice (expressing human γ-actin fused to GFP) have been described previously (Fischer et al., 2000). GFP-tKras mice were generated by injection into oocytes of a 3.4kb PvuII/PvuII fragment from pβ-actin-EGFP-last20aa K-ras4B-vector (Welman et al., 2000), in which the GFP-coding sequence is fused in frame with the 20 amino acid C-terminal plasma membrane targeting domain of K–ras4B. Transgenic animals were identified by direct inspection using a Leica MZ12 dissection microscope fitted with fluorescence optics or genotyped by PCR using GFP-specific primers.

Slice culture and microscopy

Organotypic slice cultures from P8 hippocampus were prepared as described (Gahwiler et al., 1991). For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous perfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) or with Tyrodes buffer (Gibco, Basel, Switzerland) saturated with 95% O2 /5% CO2. Imaging was performed with a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Latrunculin B (Calbiochem, San Diego, CA) was applied in ACSF at a final concentration of 2 µM. The recordings of fixation effects were made by adding 0.5% glutaraldehyde in ACSF at 37°C. Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany) and analyzed with MetaMorph software (Universal Imaging Corp., West Chester PA).

Fixation and electron microscopy

Hippocampal slice cultures were prepared for electron microscopy by microwave-assisted fixation (Jensen & Harris, 1989). Briefly, cultures were transferred into pre-warmed fixative (2% formaldehyde, 6% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer pH 7.3) and irradiated at maximum power (1000 W) for 9 sec in a Bio-Rad H2500 microwave processor. Post-irradiation temperature, measured by the built-in temperature probe, was 35 - 50° C. Hippocampal slices from 6 week-old mice were cut in ice-cold, oxygenated buffer (234mM NaHCO3, 1mM NaH2PO4, 8mM MgSO4 and 10mM glucose, pH 7.4) using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK) set at 400 µm. Slices were incubated on 0.4 µm porous culture plate insert (Millipore Corp. Bedford) for 60 min at room temperature in ACSF minus Ca2+ (117mM NaCl, 5.3mM KCl, 26mM NaHCO3, 1mM NaH2PO4, 8mM MgSO4 and 10mM glucose, pH 7.4, equilibrated with 95% O2/5% CO2) to avoid excitotoxic damage (Feig & Lipton, 1990) then at 37°C for 1.5 - 9h in ACSF. Subsequent microwave-assisted fixation was as described for slice cultures. Perfusion fixation of brain: rats (4-week-old strain RAI, n = 6) and mice (GFP-tKras, 3 x 4-week old; C57/Bl6 non-transgenic wild-type control, 6 x 8-week old, 2 x 4-week old) were anaesthetized with Rompun and Ketasol in a 0.9% saline and perfused intracardially

for 60 min with fixative (2% formaldehyde, 2.5% glutaraldehyde, 2mM CaCl2 in 0.1M CCB, pH 7.4). Dissected hippocampi were embedded in 3% agarose and 400 µm thick slices cut using a Leica VT1000s vibrating knife microtome (Leica, Glattbrugg, Switzerland). Slices were washed 5 x 10 min in 0.1M cacodylate buffer then post-fixed for 60 min 1% osmium tetroxide (Sigma, St. Louis MO) and 1.5% potassium ferrocyanide (Fluka, Buchs Switzerland) in 0.1M cacodylate buffer, pH 7.3 and block stained in 4% uranyl acetate in 70% ethanol. Serial ultra-thin sections of area CA1 were stained for 30 min with 6% aqueous uranyl acetate then for 15 min with 0.4% lead citrate in 0.04M NaOH, in an N2-gassed box.

Results

Visualization of the neuronal surface using GFP-tKras

To directly label the surfaces of dendrites in living neurons we generated lines of transgenic mice expressing a cell surface marker, GFP-tKras, consisting of GFP fused to the carboxyl-terminal 20 amino acids of the human K-ras 4B oncogene which targets GFP to the plasma membrane (Welman et al., 2000). The K-ras 4B tag sequence spreads freely in the plane of the membrane (Niv et al., 1999; Prior et al., 2001) so that the GFP-tKras marker distributes evenly over the cell surface and provides a direct and unbiased view of neuronal morphology.

In transgenic mice the GFP-tKras fusion protein was expressed throughout the brain (Fig. 1A). In the hippocampus, expression was strongest in area CA1 (Fig. 1B) and this pattern was consistent in 3 separately generated lines of mice presumably reflecting area-selective activity of the chicken β-actin promoter sequence used to drive the expression of the GFP-tKras construct (Ludin et al., 1996). Animals from any of these three lines appeared phenotypically normal when compared to non-transgenic littermates. General activity, fertility, survival to adulthood and longevity did not differ noticeably between GFP-tKras animals and non-transgenic littermates or wild-type mice. Average brain weights of randomly selected GFP-tKras animals at 2 months of age (480.0±34.6 mg, n = 7) and C57/Bl6 mice (474.2±19.88 mg, n = 7) did not differ significantly ($p = 0.71$) and no differences in cell numbers and distribution were apparent in Nissl-stained brain sections (data not shown). Altogether these results suggest that expressing the GFP-tKras marker had no significant impact on neuronal structure or function. Confocal images from acutely cut slices of hippocampus from brains of young adult (5 week-old) mice showed that expression was strongest in spiny dendrites (Fig. 1C), further reflecting selective activity of theβ-actin promoter which expresses at high levels in spine-bearing neurons (Kaech et al., 1997).

Time-lapse recordings made using acutely cut slices of hippocampus showed that motile protrusions were present on dendrites in both developing and young adult brain tissue (supplementary video data to Fig. 1C). Dendritic protrusions in slices from developing (3 week-old) hippocampus appeared as rapidly extending and retracting filopodia (Fig. 1X.mov) whereas those in slices from young adult (5 week-old) brain had the typical morphology of mature dendritic spines with a thin neck and expanded head (Fig. 1C.mov). The comparative morphology of dendrites in acutely cut slices thus reflects the progression from filopodia to the mature spine structures that occurs progressively during the first month of life after birth in rodents (Fiala et al., 1998). Despite the limited optical resolution in acute slices, these recordings also showed that heads of dendritic spines in young adult brain tissue are motile.

We next examined slices taken from developing hippocampus or cerebral cortex at postnatal day 8 (P8) and then maintained in vitro for up 3 months. This approach has the advantages that dendrite development can be monitored continuously and that optical resolution is greatly increased compared to acutely cut slices (compare Fig. 1 C and D). This improvement was particularly significant in mature slices (≥1 month in vitro), where the GFP-tKras surface marker enabled the detailed structure of dendrite shafts and spines to be resolved in live cell recordings (Figs. 1D and 3).

Developmental changes in surface motility of dendrites

Up to 2 weeks in vitro, dendrites in slices established at P8 produced large numbers of highly motile filopodia that were closely similar in appearance to those of dendrites in chronologically equivalent acute slices from 3 week-old brain (Fig. 2). Spine-like protrusions were also present on these immature dendrites but were morphologically unstable, undergoing gross shape changes and frequently "morphing" into filopodia (Fig. 2A and B). These characteristics are consistent with previous studies showing the presence of highly motile filopodia and morphologically variable "protospines" on developing dendrites (Dailey & Smith, 1996a; Ziv & Smith, 1996; Dunaevsky et al., 1999; Lendvai et al., 2000; Parnass et al., 2000; Wong & Wong, 2000; Marrs et al., 2001).

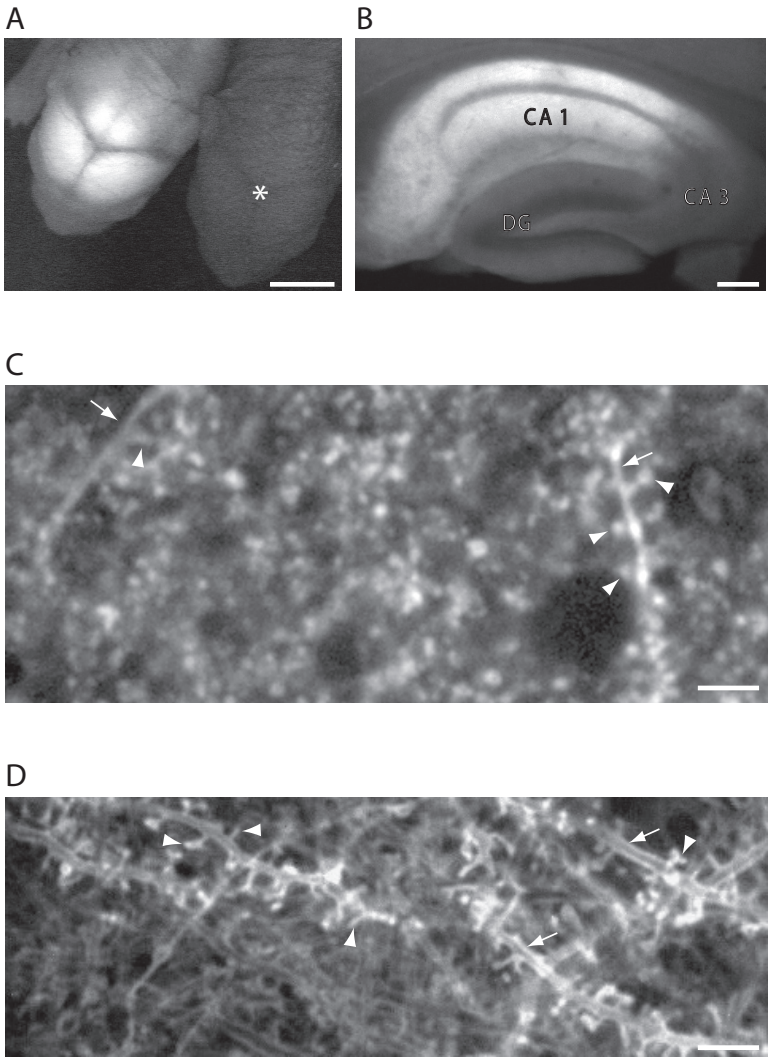


Figure 1: Visualization of dendrites and spines in transgenic mice expressing surface membrane-targeted GFP
(a) Three day old transgenic and non-transgenic (white asterisk) littermates from a line expressing GFP-tKras viewed using fluorescence optics. Bar = 0.5 cm. (b) A freshly cut 400 µm thick slice from the hippocampus of an 8 day-old old GFP-tKras transgenic mouse showing stronger GFP fluorescence in area CA1 than in area CA3 or dentate gyrus (DG). Bar = 50 µm. (c) A single frame from a time-lapse recording of the CA1 molecular layer in a hippocampal slice from a 5 week old GFP-tKras mouse. Arrows indicate dendritic shafts and arrowheads dendritic spines. Bar = 5 µm. See supplementary video data for original time-lapse recordings. (d) A single frame from a time-lapse recording of the CA1 molecular layer in a hippocampal slice culture from a GFP-tKras mouse after 6 weeks in vitro. Arrows indicate dendritic shafts and arrowheads dendritic spines. Bar = 5 µm.

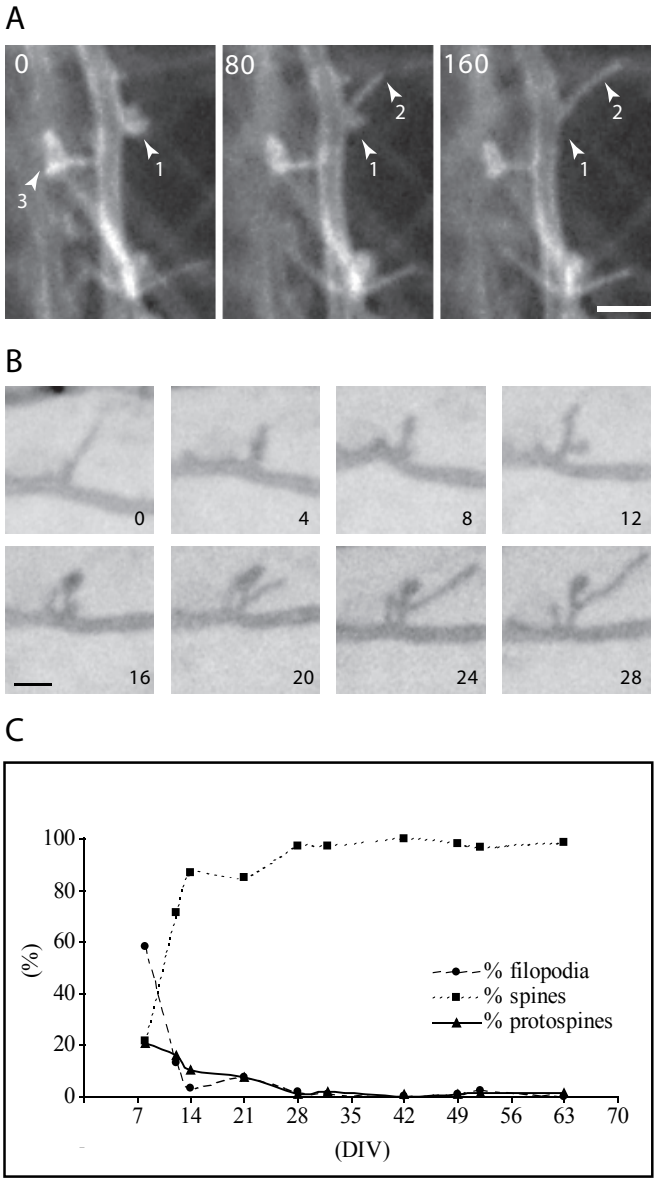


Figure 2: Maturation of dendritic protrusions in hippocampal slice cultures.
(a) Three frames from a time-lapse recording show a single dendrite in area CA1 of a hippocampal slice culture after 8 days in vitro. Time scale (top left) in seconds. Details: A stubby protrusion (1) converts into a filopodium (2) while a mushroom spine (3) retains its basic shape. Bar = 3 μm. (b) Characteristic anatomical instability of a 'protospine' in a hippocampal slice culture after 12 days in vitro. The contrast has been inverted for clarity. Time scale in minutes, bar = 2 μm. See supplementary video data for original time-lapse recordings. (c) Relative proportions of filopodia, 'protospines' and spines in hippocampal slices during development in culture. Results from 1902 dendritic protrusion in 19 independent slice cultures (see supplementary data Table 2X).

Dendrites continued to produce filopodia and protospines at decreasing levels until 28 days in vitro after which time they were only rarely encountered (Fig. 2C).

By contrast after developing for 1 month or more in vitro dendrites in GFP-tKras slices produced dendritic spines of mature appearance showing the three morphological categories - stubby, mushroom and thin (Fig. 3A and B) - conventionally recognized in adult brain (Peters & Kaiserman-Abramof, 1970; Spacek & Hartmann, 1983; Harris et al., 1992). Instead of the gross shape changes characteristic of protrusions from developing dendrites, mature spines from slices of both hippocampus (n = 43) and cerebral cortex (n = 18) were fundamentally stable and retained the same basic shape when observed for periods of up to 6 h (Fig. 3B). Gross changes of morphology in these mature dendrites were infrequent: in time-lapse recordings of 30 min duration made from 539 spines of 8 independently established cultures, 8 spines showed unequivocal changes in length of the spine neck, there were 6 filopodia (headless protrusions undergoing repetitive extension and retraction) and 1 new spine arose from a dendrite. Thus the maturation of dendritic spines from unstable filopodial precursors to fundamentally stable structures of adult morphology can be followed using brain slices maintained in vitro.

Table 1. Relative abundance of dendritic spine types

Location	Age	% m	% t	% s	% i	Reference
Hippocampus	DIV 30	51 ± 11	25 ± 4	24 ± 11	-	Roelandse 2003
Barrel Cortex	P12	57 ± 3	15 ± 2	30 ± 3	-	Lendvai 2000
Hippocampus	DIV 14	24 ± 6	34 ± 4	42 ± 5	-	McKinney 1999
Hippocampus	P15	28	37	35	-	Spacek 1997
	Adult	31	59	4	-	
Hippocampus	P15	22 ± 17	17 ± 16	39 ± 29	-	Harris 1992
	Adult	19 ± 11	62 ± 18	4 ± 7	-	
Visual Cortex	Adult	17	55	7	17	Spacek 1983*
Visual Cortex	Adult	9 ± 4	72 ± 2	19 ± 4	-	Peters 1970

The numbers of spines, categorized by shape as mushroom (m), thin (t) or stubby (s) (Peters et al. 1970), in 4 organotypic hippocampal slice cultures were counted (top row). Results from previous publications are shown for comparison. Note the high abundance of mushroom spines between the two studies using live cell imaging (top two rows) compared to studies of fixed tissue. *) This study uses an additional classification of intermediate (i) spines defined as a morphology intermediate between m and t.

Focal actin-based motility is restricted to spine heads

Dendrites in tissue from GFP-tKras mice were labelled over the entire surface, outlining both dendrite shafts and spines as would be expected for a general plasma membrane marker (Fig. 4A, left). In previous studies we demonstrated that dynamic actin filaments in the heads of dendritic spines can be visualized in neurons expressing actin-GFP (Fig. 4B, left) see (Fischer et al., 1998). Thus by making time-lapse recordings of hippocampal slices from actin-GFP and GFP-tKras transgenic mice maintained in culture for ≥1 month (Fig. 4) we could relate surface motility in dendrites to their underlying actin dynamics. The right-hand panels in Fig. 4A and B show motility profiles prepared from these time-lapse recordings by summing between-frame differences in pixel grey-scale densities for 60 frames taken 10 sec apart. These are displayed using a pseudo-colour scale in which motile regions appear red to white whereas non-motile areas are blue (Fischer et al., 2000). With GFP-tKras as a marker the highest motility signals occurred at the ends of spines (arrowheads, Fig. 4A, right). Since the fluorescent signal from GFP-tKras labels the entire surface of the dendrite (Fig. 4A, left) this indicates that motility is especially high at the distal tips of spines, a conclusion which is further supported by the high dynamic activity visible at spine tips in time-lapse recordings of dendrites expressing either actin-GFP (see Fischer et al., 1998; Fischer et al., 2000) or GFP-tKras. We have shown previously that drugs which inhibit actin dynamics block actin motility in spines (Fischer et al., 1998). The same drugs (cytochalasin D 1.6 µg/ml, data not shown; latrunculin B 2 µM, Fig. 4C) blocked spine motility visualized using GFP-tKras, demonstrating that the surface motility of dendritic spines is dependent on actin dynamics.

Table 2x: Relative abundance of dendritic protrusions

n	DIV	% F	% S	% P
317	8	0.580	0.215	0.205
348	12	0.129	0.713	0.158
209	14	0.029	0.871	0.100
180	21	0.074	0.852	0.074
105	28	0.019	0.971	0.010
111	32	0.009	0.973	0.018
49	42	0.000	1.000	0.000
231	49	0.009	0.983	0.009
156	52	0.019	0.968	0.013
196	63	0.000	0.990	0.010

Relative proportions of filopodia (% F), 'protospines' (%P) and spines (% S) in hippocampal slices during development in culture. Indicated are days in vitro (DIV), the total number of protrusions counted per time point (n) and percentages (%). Results from 1902 dendritic protrusion in 19 independent slice cultures

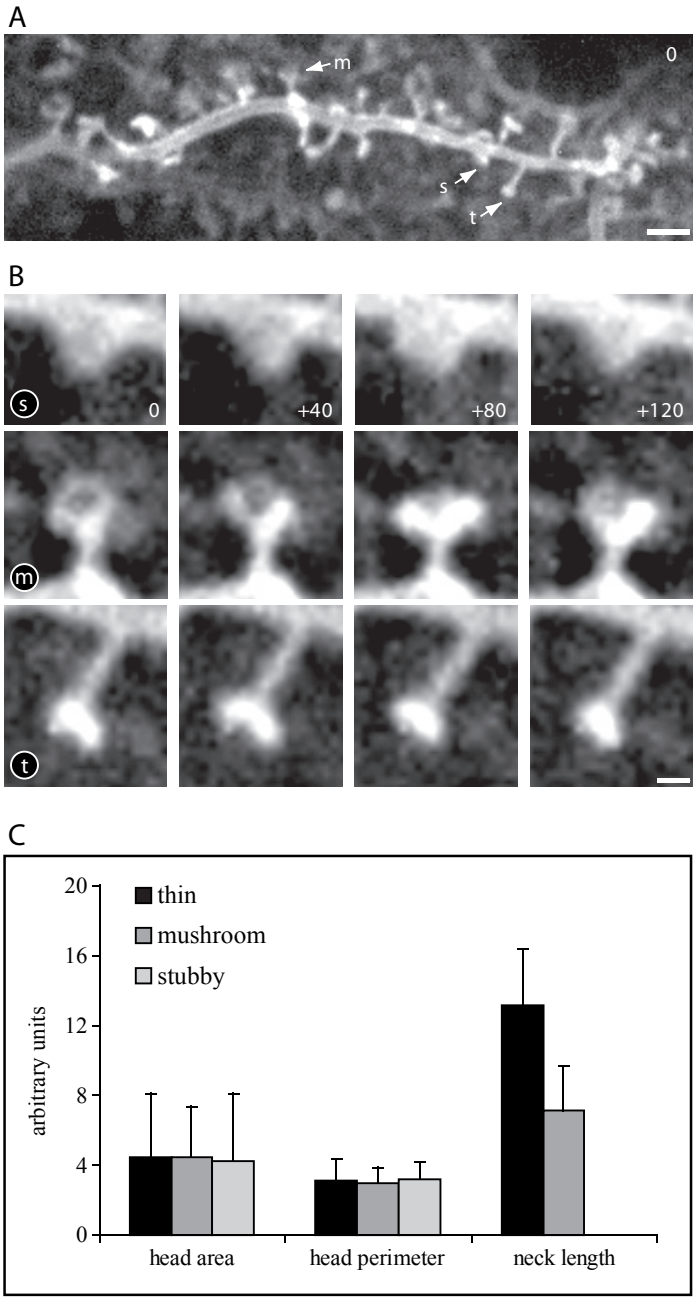


Figure 3 Dendritic spine types characteristic of adult brain are visible in live hippocampal slice cultures from GFP-tKras mice.
(a) The first frame from a 10-minute time-lapse recording (60 frames) of confocal images from a single dendrite in an 8 week-old GFP-tKras slice culture. Labeling of spines corresponds to the classification of Peters et al. (1970): stubby (s), mushroom (m), and thin (t). Bar = 2 μm. (b) Detailed views of s, m and t spines showing, for each case, four frames taken 40 sec apart. Morphological variation is marked in mushroom-shaped spines (m). Bar = 0.5 μm. See supplementary video data for original time-lapse recording. (c) Measurements of spine area (left) and perimeter (middle) show no differences between the 3 spine categories (data taken from time series of 131 spines in 4 independent cultures) whereas neck length (right) differs significantly between thin and mushroom spines (stubby spines are defined as having motile heads but no neck. Data from 184 spines in 6 independent slice cultures).

Motile lamellipodia produce cup-shaped spines partially enclosing axonal boutons

In living GFP-tKras slice cultures, counts of the numbers of stubby, thin and mushroom spines showed a far higher proportion of "mushroom" profiles than had been reported in previous studies based on fixed tissue (Table 1). Instead, the proportion of mushroom-shaped spines in GFP-tKras slices matured in vitro (52 ± 10%) is similar to that reported for dendritic spines in living brain (57 ± 3% in Lendvai et al., 2000) and is approximately double the average value for mushroom spines reported in earlier studies on fixed tissue (Table 1). However, time-lapse recordings of GFP-tKras slices suggest that these counts are misleading because all spines had motile lamellipodia-like extensions so that at some times spine head appeared mushroom-shaped and at others more rounded. Supporting this interpretation, measurements of the area and perimeter of spine heads from live cell images showed no significant variation between spines of the three conventional categories (Fig. 3C). Instead the primary morphological difference between "thin", "mushroom" and "stubby" spines lay in the

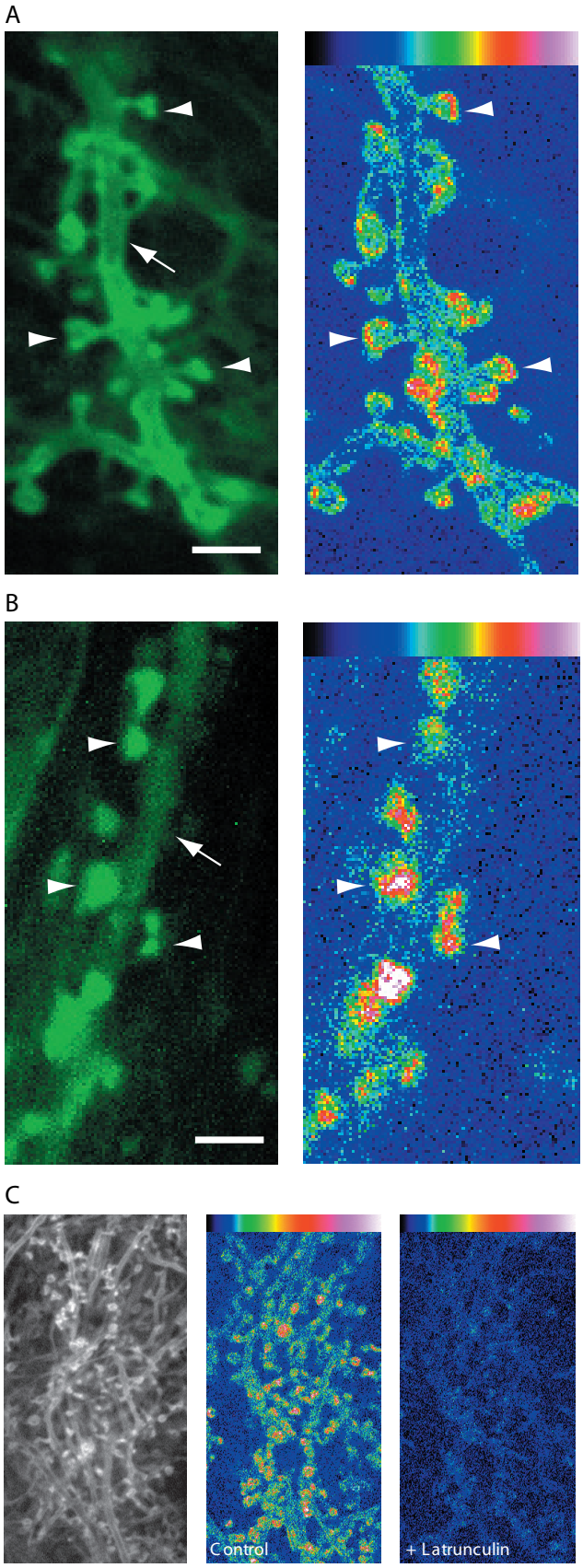


Figure 5: Dendritic spine ultrastructure in mature hippocampal slice cultures.
(a) Electron micrograph of the molecular layer of area CA1 in a mature slice culture. Asterisks indicate axonal boutons that are partially enveloped by cup-shaped dendritic spines. Note the overall compact tissue structure and well-preserved cell morphology typical of microwave-fixed hippocampal slice cultures. Bar = 1 µm. (b) A single cup-shaped dendritic spine (sp) and its axonal bouton (b) from the boxed area in panel (a). Note the lamellipodial extension from the spine head partially enclosing the axonal bouton. Bar = 0.5 µm. (c & d) Further examples of cup-shaped spines investing presynaptic boutons in 4-week old slice culture.

length of the spine neck which was respectively long, intermediate or absent (Fig. 3B and C).

To determine whether motile spine heads seen in GFP-tKras slice cultures represent bone fide synapses, we fixed slices after time-lapse imaging and examined them by electron microscopy. As controls we examined tissue from both wild-type animals and non-transgenic littermates to the GFP-tKras mice that had been main-

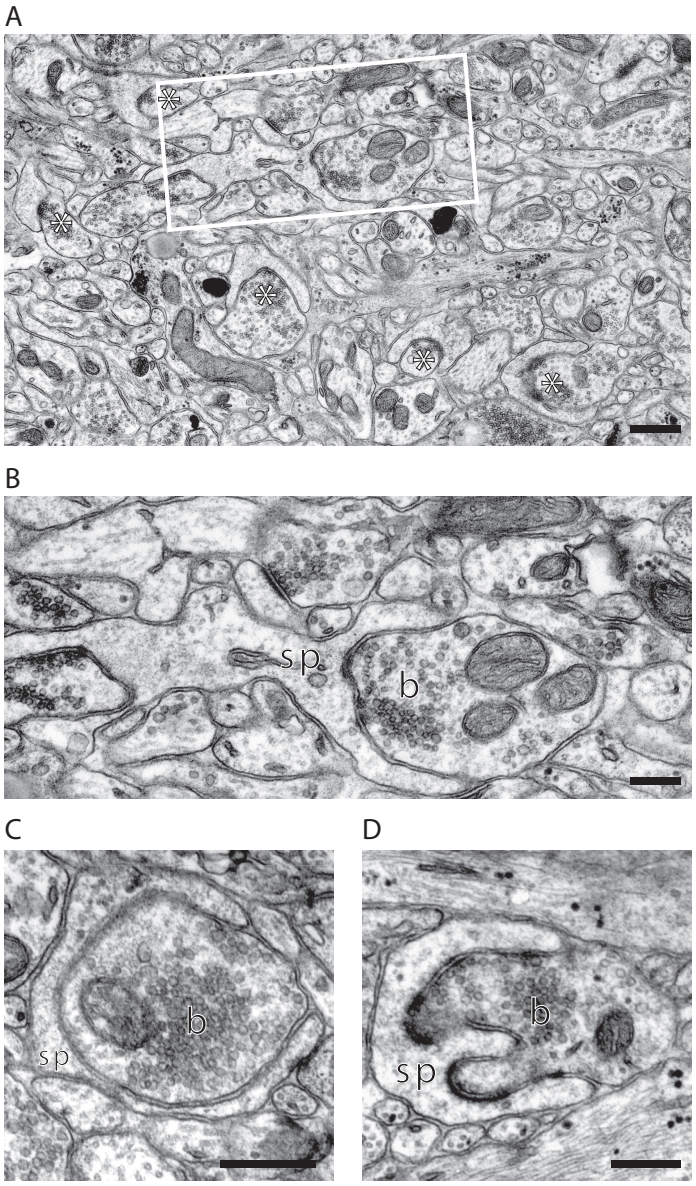


Figure 6: Cup-shaped dendritic spines in mature brain
(a) Neuropil ultrastructure of area CA1 in slices from 6 week-old mouse hippocampus prepared using microwave-enhanced fixation. Cup-shaped dendritic spines partially enclose axonal boutons (*). Bar = 0.5 µm. (b) Examples of spine synapse profiles used to quantify cup-shaped and round spines. 1 & 2 round; 3 & 4 lower limit cup-shaped; 5 & 6 extreme cup-shaped spines. Bar = 0.2 µm. (c) Effects of fixation procedure on preservation of cup-shaped spine morphology. Round and cup-shaped spines were classified according to the criteria described in B in 250 µm2 fields from perfusion-fixed brain ("perfused"; n = 5) and microwave-fixed brain slices ("MW acute"; n = 7), both from 6 week-old mice, and 5 week-old microwave-fixed hippocampal slices cultures ("MW culture"; n = 4). Note the relative abundance of cup-shaped spines in tissue prepared using microwave-assisted fixation as compared to perfusion-fixed tissue. *$p < 0.05$, **$p < 0.01$ (two-tailed Student's t-test).

tained in vitro for equivalent periods. Slices from all three sources were poorly preserved when fixed by immersion in conventional electron microscopy fixatives consisting of mixed aldehydes (Reese & Karnovsky, 1967) and showed expanded extracellular space suggestive of cell shrinkage (data not shown). By contrast, slices fixed using a rapid, microwave-assisted procedure (Login, 1983; Jensen & Harris, 1989) showed well-preserved cell morphology and overall compact tissue structure comparable to that of well-fixed intact brain (Fig. 5A, B). Within microwave-fixed slices cup-shaped spines with lamellipodia-like protrusions partially enclosing presynaptic boutons were common (Fig. 5A). The cup-shaped appearance of these spines was comparable to that of mushroom spines visualized by live cell imaging in GFP-tKras slices (compare Figs. 5B to D to Fig. 3B, middle row).

To compare spine morphology in brain slice cultures with that of intact brain tissue, we examined acutely cut slices from hippocampi of normal, non-transgenic mice prepared for electron microscopy using microwave-assisted fixation (Fig. 6). In area CA1 from 6 week-old animals prepared using this method, cup-shaped spines with lamellipodia extending around presynaptic boutons were common (asterisks in Fig. 6A). To compare the

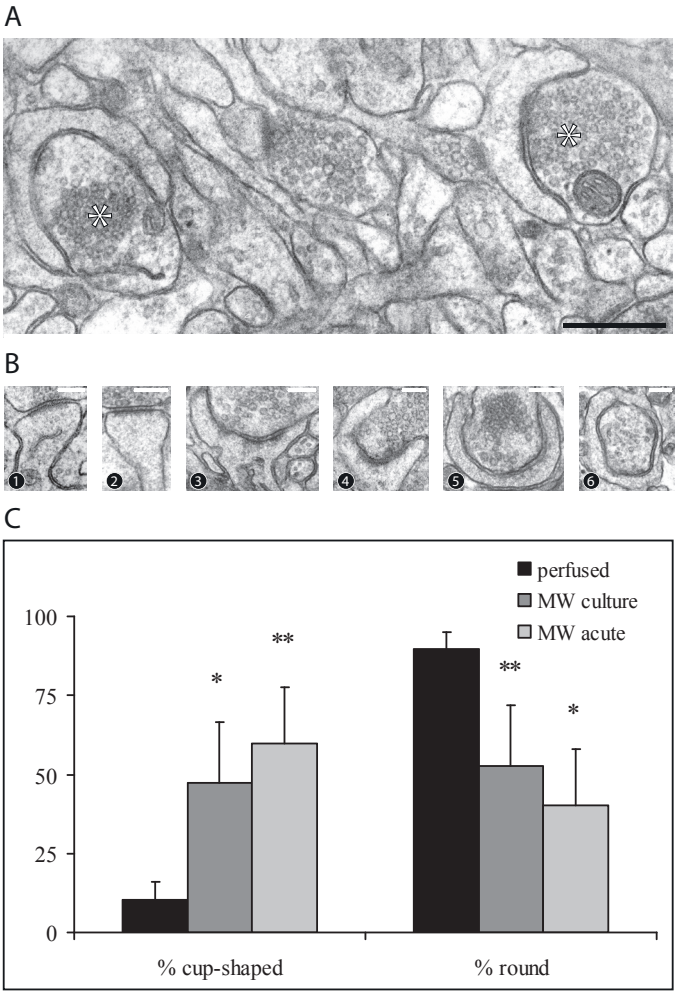
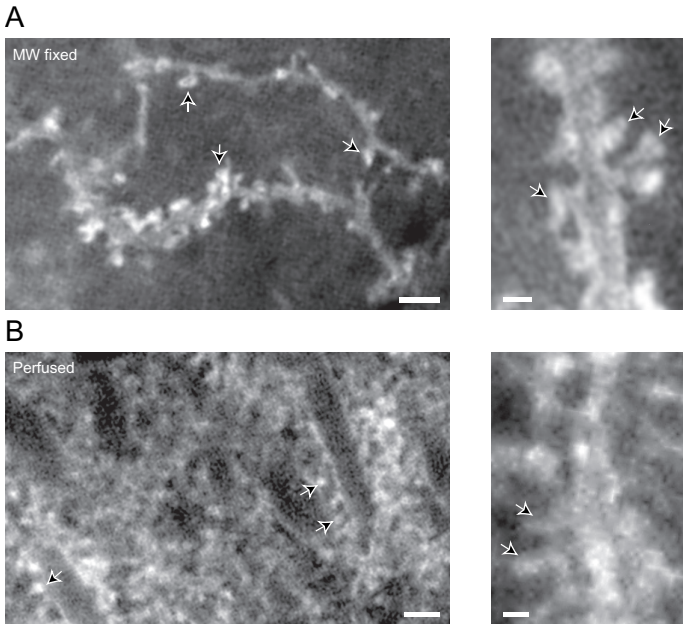


Figure 7: Comparison of morphological preservation in tissue prepared using microwave-assisted or conventional perfusion fixation.
(a) Confocal fluorescence images of area CA1 in hippocampal slice cultures from GFP-tKras transgenic mice prepared using microwave-assisted fixation. Bars: left = 3 µm, right = 1 µm. (b) Equivalent micrograph of slice culture from GFP-tKras expressing hippocampus fixed by immersion. Arrows indicate spines. Bars: left = 3 µm, right = 1 µm.

relative abundance of round and cup-shaped spines, we performed single section counts using the morphological criteria shown in Fig. 6B. This revealed that in microwave-fixed tissue, cup-shaped and round spines were similarly abundant (Fig. 6C). By contrast, in tissue prepared by perfusion, round spines were over 8–fold more abundant than cup-shaped spines (Fig. 6C). One reason for the difference between microwave-fixed brain slices and perfusion-fixed brain might be the difference in speed of fixation. To examine this possibility we used confocal microscopy to record the effect on neuronal structure of fixation by immersion in 0.5% glutaraldehyde. In hippocampal slice cultures expressing either GFP-tKras (n = 4) or actin-GFP (n=2) this revealed extensive tissue shrinkage and rounding up of spines as the slices were fixed (Fig. 7).

Discussion

Growing evidence that dendritic spine geometry is a major determinant of synaptic transmission at excitatory synapses (Takumi et al., 1999; Matsuzaki et al., 2001) underlines the importance of precisely characterizing spine shape and its variation in living neurons. However, although previous studies have demonstrated that mature dendritic spines with synaptic contacts are morphology plastic (Fischer et al., 1998; Dunaevsky et al., 2001; Grutzendler et al., 2002; Trachtenberg et al., 2002), the extent of this plasticity has remained poorly defined, largely because methods for resolving the fine structure of living dendrites were lacking. The transgenic mice described here are particularly useful for this purpose because the plasma membrane-targeted GFP-tKras marker intrinsically expressed in their neurons directly reveals the configuration of the cell surface. Another



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advantage of this approach is that the tissue used for live cell imaging is derived from phenotypically normal animals thus avoiding potential artefacts introduced by injecting or transfecting markers into cells. Time-lapse recording of dendritic spines in mouse brain with sufficient spatial and temporal resolution to determine their dynamic state have not yet been reported. Furthermore, recordings from anesthetized animals will probably underestimate spine motility since anaesthetics have been shown to block actin dynamics in dendritic spines at clinically effective concentrations (Kaech et al., 1999).

The character of dendrite plasticity changes during development

While confirming fundamental aspects of past studies, our results reveal significant new features of dendritic spine morphology and plasticity. In agreement with previous evidence, GFP-tKras labelled neurons show that depending on their stage of development dendrites generate two distinct kinds of motile protrusions. Those formed early in development are morphologically unstable, appearing and disappearing within minutes and transforming rapidly from filopodia to irregularly shaped "protospines" (see Dailey & Smith, 1996; Ziv & Smith, 1996; Parnass et al., 2000; Wong & Wong, 2000). The distinguishing characteristic of these immature protrusions is that shape changes occur over their entire surface. By contrast, mature dendritic spines, as shown by GFP-tKras imaging of both acutely cut and in vitro maintained brain slices, are fundamentally stable structures in which morphological plasticity is essentially limited to the tip of the spine head (Fig. 4). The distinction between these two modes of plasticity is particularly clear when time-lapse recordings of immature and mature dendrites are compared.

The motile filopodia of immature dendrites have been widely discussed as representing a mechanism of searching the developing neuropil for potential synaptic partners (Dailey & Smith, 1996; Jontes & Smith, 2000; Wong & Wong, 2000). Ideas regarding the functional significance of morphological plasticity in mature dendritic spines are far less developed. One reason for this is that the term "plasticity" has been used inconsistently in different studies to variously describe changes in spine number (Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999), in spine length (Lendvai et al., 2000; Korkotian & Segal, 2001; Marrs et al., 2001) or in spine shape

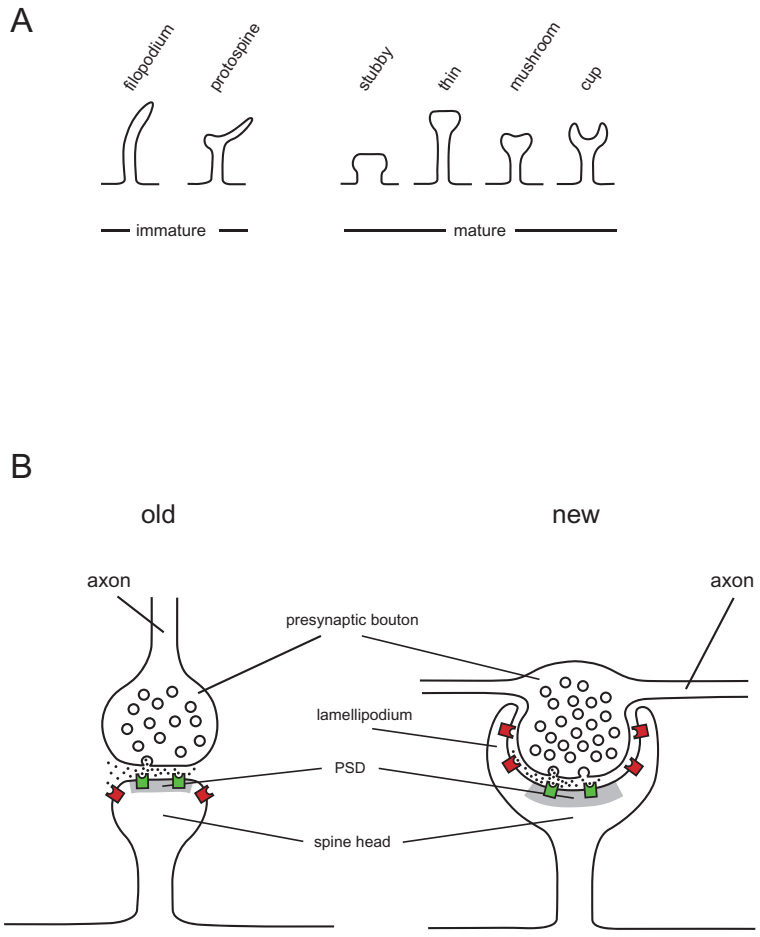


Figure 8: Configuration of round and cup-shaped dendritic spines.
Diagrams comparing the conventional image of "round" dendritic spines to the lamellipodial "cup-shaped" spines described in this study. The comparative distribution of junctional receptors (green) and extra-junctional receptors (red) implied by the two configurations is indicated. Black dots represent the spread of presynaptically released neurotransmitter, indicating the difference in synaptic cleft path-length in the two cases.

(Fischer et al., 1998; Dunaevsky et al., 1999). In part this reflects diverse concepts of morphological plasticity at the synapse. However, another contributory factor to this uncertainty is that the apparent motility of dendritic spines can vary depending on the conditions used for time-lapse recording. Recordings made at rates of around one frame per minute are well suited to demonstrating changes in the number or length of unstable protrusions on immature dendrites (Dailey & Smith, 1996; Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Marrs et al., 2001), but fail to capture the more subtle morphological plasticity of mature spines, which is only evident in recordings made at frame rates in the order of seconds (Fischer et al., 1998; Dunaevsky et al., 1999). Indeed changes in the shape of spine heads are visible in frames taken 1.5 sec apart (Fischer et al., 1998) and in "streamed" recordings where frame are captured at 0.5 sec intervals (U. Wagner, unpublished observations).

Spatial restriction of dynamic actin filament distribution

The focal location of morphological plasticity at the tips of mature dendritic spines is consistent with its dependence on dynamic actin filaments, which are concentrated at the periphery of spine heads (Fischer et al., 1998; Fischer et al., 2000). This relationship is confirmed by the effect of latrunculin B and cytochalasin D which suppress spine actin dynamics (Fischer et al., 1998) and also block spine tip motility visualized using the GFP-tKras surface marker (Fig. 4C). The restricted distribution of actin-based motility in mature dendritic spines compared to their filopodial precursors also agrees with evidence that the actin cytoskeleton becomes progressively more stable as synaptic structure matures (Zhang & Benson, 2001). On the other hand a recent

study using photobleach recovery of actin-GFP fluorescence to measure actin dynamics demonstrated that a major fraction of actin filaments in mature dendritic spines continues to turn over rapidly (Star et al., 2002). Our results, showing that mature dendritic spines contain highly dynamic but spatially restricted actin filaments, are consistent with both sets of data. The presence of both stable and dynamic actin filaments in the spine cytoplasm supports a model in which a corona of dynamic actin at the spine periphery is superimposed on a more stable core of actin filaments (Fischer et al., 1998; Halpain, 2000; Matus, 2000), a pattern that is consistent with electron microscopic analysis of cytoskeletal structure in the spine cytoplasm (Landis & Reese, 1983).

Cup-shaped dendritic spines are abundant on living dendrites

In hippocampal slice cultures from GFP-tKras transgenic mice the maturation of dendritic spine morphology was not complete until 1 month in vitro. Prior to that time morphologically unstable filopodia and protospines were still common whereas afterwards they were extremely rare. This time-scale of maturation agrees with other studies of dendrite development in rodent hippocampal slice cultures (Dailey & Smith, 1996; Collin et al., 1997; Nakayama et al., 2000) and the developing brain (Fiala et al., 1998). Following this transition there was no further change in basic morphology up to 3 months in vitro, indicating that the state reached after 1 month represents a stable endpoint for dendrite structure. In this mature state, the dendritic spines visible in GFP-tKras images were of four types. Three of these correspond to the categories – stubby, mushroom and thin – conventionally recognized in adult brain (Peters & Kaiserman-Abramof, 1969; Spacek & Hartmann, 1983; Fiala et al., 1998). The fourth variety consisted of cup-shaped spines characterized by a motile lamellipodia-like expansion of the spine head enclosing the presynaptic axon terminal to varying degrees. Although cup-shaped spines may constitute an additional independent category they may instead represent the true in vivo morphology of the previously recognized mushroom spine category. In addition to their motility, the conclusion that the heads of these cup-shaped spines are fundamentally lamellipodial is consistent with evidence that the structural maintenance of mature dendritic spines depends on the small G protein rac (Luo et al., 1996; Nakayama et al., 2000; Nakayama & Luo, 2000) which is selectively involved in lamellipodial function of the actin cytoskeleton (Nobes & Hall, 1995; Van Aelst & D'Souza-Schorey, 1997; Ridley, 2001).

In GFP-tKras confocal images of living slice cultures 52%±11 of spines were mushroom–shaped, a level similar to the 47±19% found in electron micrographs of microwave-fixed slices. This is markedly different from the proportion of cup-shaped spine in perfusion-fixed tissue from 4 week-old mouse hippocampus where only 12% of spines were cup-shaped, a value in the same range reported in previous studies using conventional electron microscopy procedures (Table 1). One explanation for this disparity might be that spine morphology is inadequately preserved by perfusion fixation with aldehydes, which penetrate tissue slowly (Hopwood, 1967). Indeed slices fixed by immersion in aldehydes were poorly preserved compared to those prepared using rapid, microwave-assisted fixation, a result which agrees with the improved preservation of various tissue including brain reported for this method (Login, 1983; Jensen & Harris, 1989). This difference may reflect fixation sensitivity of the actin-rich lamellipodia of cup-shaped spines since time-lapse recordings made during glutaraldehyde fixation showed spines shrinking and rounding up (supplementary video data Fig. 7x). Actin-rich structures are known to be fixation sensitive (Maupin-Szamier & Pollard, 1978; Small, 1981; Slepecky & Ulfendahl, 1988; Small et al., 1999; Dino & Mugnaini, 2000) and in at least one other case microwave-accelerated fixation has

been found to improve their preservation (Sawitzky et al., 1996).

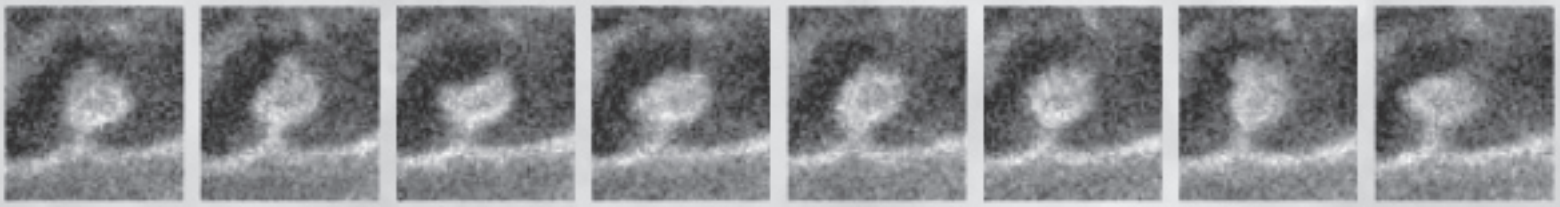
Functional implications of dendritic spine morphology and plasticity

What then is the role of actin-based motility in mature dendritic spines? One widely discussed idea is that it represents the molecular basis for changes in synaptic connectivity involved in learning and memory (Crick, 1982; Halpain, 2000; Matus, 2000). This hypothesis is supported by evidence linking manipulations of sensory input and learning paradigms to alterations in spine morphology and motility (for recent examples see Klintsova & Greenough, 1999; Lendvai et al., 2000; Geinisman et al., 2001; Knott et al., 2002). Experiments showing that drugs which block actin polymerization interfere with LTP (Kim & Lisman, 1999; Krucker et al., 2000) also implicate dynamic actin filaments in learning-related synaptic plasticity.

The rapid changes in dendritic spine shape revealed by GFP-tKras imaging further suggest a possible role for actin dynamics in fine tuning several parameters of synaptic transmission at glutamatergic synapses (Fig. 8). For example, spine morphology is thought to influence the duration and spread of postsynaptic Ca2+ currents (Koch & Zador, 1993; Markram & Sakmann, 1994; Eilers et al., 1995; Svoboda et al., 1996; Yuste et al., 2000) and the distribution of postsynaptic glutamate receptors (Baude et al., 1995; Bernard et al., 1997; Lujan et al., 1997), a possibility is also suggested by recent data showing a direct relationship between spine geometry and the expression of AMPA and NMDA receptors (Ottersen & Landsend, 1997; Matsuzaki et al., 2001). Stimulation of AMPA and NMDA receptors induces reversible retraction of motile protrusions from spine heads (Fischer et al., 2000) suggesting that a feedback mechanism may exist that modulates expression of postsynaptic glutamate receptors. The morphology of cup-shaped spines also has implications for spillover, the process by which neurotransmitter release from one synapse activates receptors at another (Clements, 1996; Barbour & Häusser, 1997; Isaacson, 2000; Kullmann, 2000). Glutamate released at synapses with cup-shaped spines would encounter an extended extra-junctional contact zone containing glutamate receptors and transporter uptake sites which are considered significant in determining spillover characteristics (Clements, 1996; Barbour & Häusser, 1997).

Finally, it is tempting to speculate that the switch from the polymorphic and highly variable structure of immature protospines to the intimate contact and focal plasticity represented in the morphology of cup-shaped spines on mature dendrites may be functionally related to the focusing of circuit properties exemplified by the narrowing of CA1 pyramidal cell place fields that accompanies maturation of the hippocampus (Martin & Berthoz, 2002).

AGE-DEPENDENT EFFECTS ON NETWORK FORMATION IN ORGANOTYPIC CULTURES



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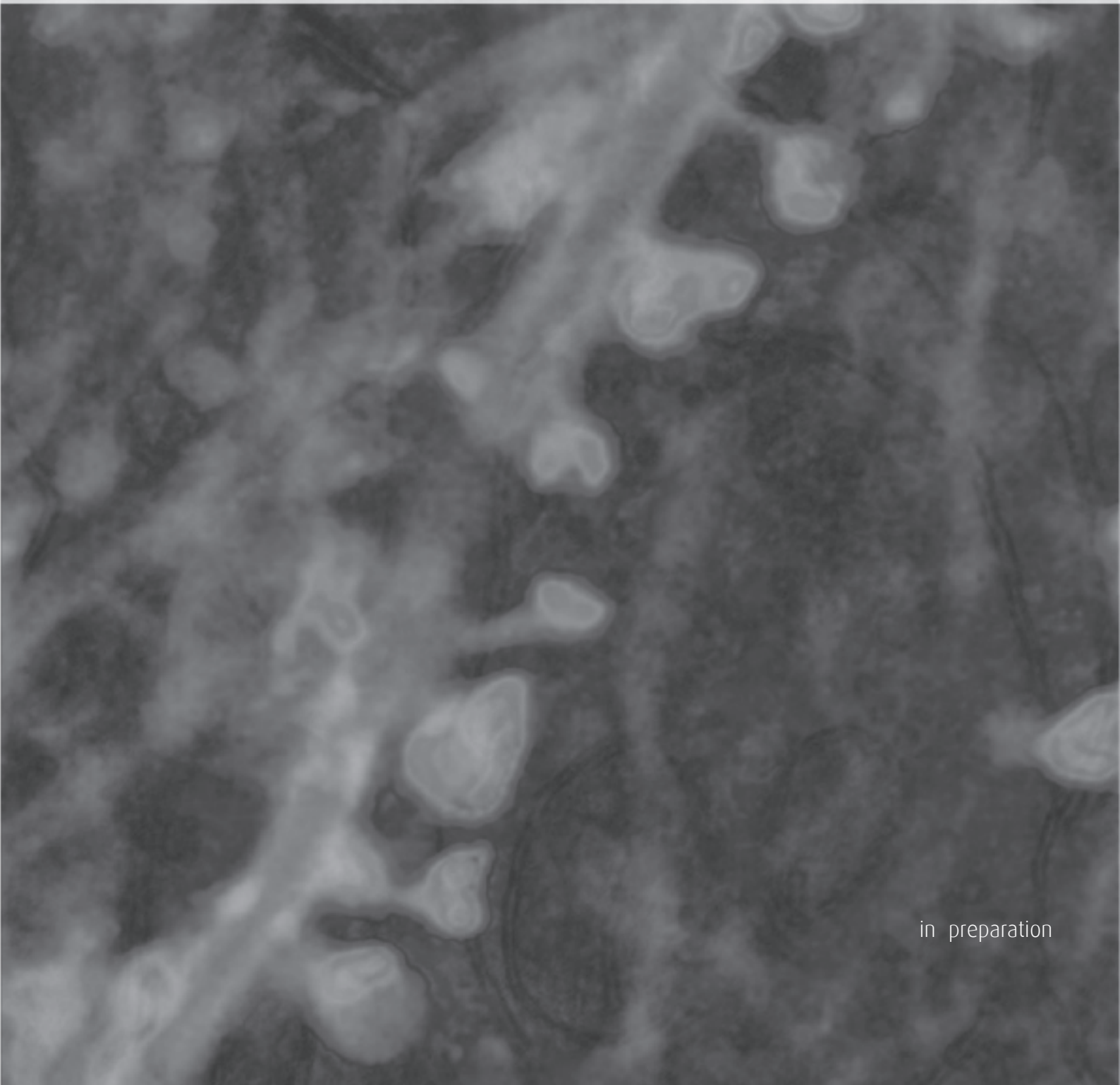


in preparation

Abstract

To explore the mechanisms underlying the establishment of neuronal connectivity and synaptic consolidation, we used co-cultures of fluorescent and non-fluorescent hippocampal slices. We show that both embryonic and early postnatal neurons are able to form axonal projections that invade the neighbouring slice in the first two weeks in culture suggesting this to be an age-independent intrinsic ability. The neuronal capacity for consolidating synaptic connections reduced dramatically in co-cultures derived from postnatal tissue resulting in the near complete absence of axons and axonal varicosities in mature cultures. In contrast, E18 neurons were able to form dense axonal cross-innervation in mature co-cultures derived from embryonic tissue and axonal varicosities along their shafts were highly dynamic, exhibiting both changes in shape and diameter and rapidly extending and retracting protrusions from the top of the varicosity. Heterochronic co-cultures of embryonic and early postnatal hippocampus resulted in progressive cell death of P8 neurons and the dramatic regression of the entire P8 tissue slice. Taken together, using co-cultures to study neural network formation in vitro and membrane dynamics of mature varicosities, our data suggests the existence of a developmental switch between the late-embryonic and postnatal period that restraining the sustained afferent cross-innervation between postnatal brain slices and causing miscommunication between postnatal and embryonic hippocampus slices leading to profound degeneration of P8 tissue. The thick layer of microglia surrounding P8 tissue suggests their involvement in neuronal degeneration as observed in axotomy-induced neuronal death and various neurodegenerative diseases such as Alzheimer. The observed membrane dynamics on mature axonal varicosities suggest a retained capacity for experience-dependent fine-tuning e.g. during either periods of learning and memory or during brain damage resulting in an altered connectivity for both pre- and postsynaptic compartments in the mature mammalian CNS.

Key Words: axonal varicosities, synaptic plasticity, neuronal development, microglia, morphology, hippocampus, time-lapse microscopy, green fluorescent protein

Introduction

One of the most fundamental features of neurons is their polarized organization with two types of neurites extending from the cell body, axons and dendrites that are both functionally and morphologically distinct From early in development on, neurons are polarized cells that have an (Bradke & Dotti, 2000). Axons travel long distances and are guided along specific pathways to their targets by attractive and repulsive cues (Mueller, 1999; Song & Poo, 2001; Dickson, 2002). In general, dendrites do not extend as far as axons from the cell body but instead branch extensively, giving rise to a dendritic tree whose development is guided by numerous factors, each of them regulating different decision points along the developmental path, whether to branch, turn or stop (Cline, 2001; Scott & Luo, 2001; Whitford et al., 2002). In the developmental state, both axons and dendrites possess growth cones and filopodia extending from their shafts, both of which are highly dynamic and actin-rich structures (Dailey & Smith, 1996; Wong & Wong, 2000; Chang & De Camilli, 2001; Roelandse et al., 2003). The role of these structures is thought to be to sample the extracellular environment for potential soluble or membrane-bound guidance cues during initial growth and subsequently for pre- or postsynaptic partners (Jontes & Smith, 2000). Once both types of neurites have found their appropriate targets, synapses are formed in abundance. It has recently been shown that the entire process of synapse formation would occur within several hours (Ahmari et al., 2000; Friedman et al., 2000). Still, many of them subsequently change in size, move laterally along the dendritic shaft or disappear entirely within several minutes (Marrs et al., 2001) thereby permitting synaptic connection between neurons to change rapidly during early development. These observations suggest that synaptogenesis is a very dynamic process of continuous formation and elimination (Jontes & Smith, 2000; Cohen-Cory, 2002).

Others and our results have shown the advantage of using organotypic slice cultures from various regions of the brain to examine neuronal development since the in vivo sequence of developmental events is closely simulated in these cultures (e.g. hippocampus: Frotscher, 1992; Roelandse et al., 2003; or spinal cord: Rothstein et al., 1993; Takuma et al., 2002). An effective means of exploring the mechanisms underlying the establishment of neuronal connectivity is the use of co-cultured organotypic slices to study neuronal path finding (e.g. Gahwiler, 1981; Bolz et al., 1990; Molnar & Blakemore, 1991; Plenz & Kitai, 1996; Baker & van Pelt, 1997). Results of such studies suggested that neurons possess an intrinsic capacity to make the layer-specific connections and hence to simulate axonal path finding in vitro (Frotscher & Heimrich, 1993; Molnar & Blakemore, 1999). However once the developmental period of axonal path finding has passed, ingrowing axons in hippocampal organotypic cultures loose the ability to successfully innervate suggesting an age-dependent impairment of axonal outgrowth (Woodhams et al., 1993; Stoppini et al., 1997). During maturation, axonal networks are intensively remodelled; axonal arbours of the Schaffer collaterals in hippocampal organotypic cultures rapidly increase in size and number of branches during the first two postnatal week but axon branches are then subsequently reduced to half the values until the neurons are fully matured suggesting the increase in axonal complexity during early postnatal life to be a temporary one (Gomez-Di Cesare et al., 1997).

In adult animals neurons have formed axonal varicosities. The majority of these swellings along the axonal shaft hold one or more active zones where synaptic vesicles fuse with the plasma membrane to release neurotransmitter (Shepherd & Harris, 1998). Varicosities are, despite differences in size and shape, organized in a

random distribution within the different brain areas (Shepherd et al., 2002) and face the postsynaptic density containing the various neurotransmitter receptors. As for their postsynaptic partners, axonal varicosities have been shown to change in numbers upon either stimulation or inhibition (Hatada et al., 2000; Colicos et al., 2001; Luthi et al., 2001; De Paola et al., 2003) and have hence been proposed as a morphological substrate for synaptic plasticity. Furthermore axonal varicosities have been associated with the presence of an actin-cytoskeleton that is thought to be involved in neurotransmitter release (Morales et al., 2000; Sankaranarayanan et al., 2003) but it is so far unclear whether this actin-based cytoskeleton can influence morphological changes in axonal varicosities to a similar extent to those observed at a sub-seconds' timescale in dendritic spines (Fischer et al., 1998).

To address this question, we studied the process of network formation using fluorescently tagged and non-fluorescent organotypic cultures of both embryonic and early postnatal animals. We found that neurons in both P8-P8 and E18-E18 co-cultures initially formed axonal growth cones that would invade at first a neighbouring slice however very few neurons in P8-P8 co-cultures were able to preserve these axonal projections that initially extended throughout the other culture. By contrast, neurons in E18-E18 co-cultures developed a dense network of axonal processes that persisted over several weeks and where still present after 54 DIV. Co-culturing slices of P8-E18 hippocampus revealed an unexpected phenomenon in which there was progressive cell death of P8 neurons and dramatic regression of the entire P8 tissue slice.

Methods

Slice culture and microscopy

Experiments were performed with transgenic animals expressing either a membrane-targeted form of GFP to label the entire neuronal surface (GFPtKras, Roelandse et al., 2003) or actin-GFP to visualize the actin cytoskeleton (Fischer et al., 2000). Organotypic slice cultures from P8 hippocampus were prepared as described (Gahwiler et al., 1998). Organotypic slice cultures from E18 hippocampus brains were prepared as follows. Transgenic mouse embryos were identified by direct inspection using a Leica MZ12 dissection microscope fitted with GFP-optimized fluorescence optics. Brains were dissected in ice-cold Gey's balanced salt solution (GBSS - Gibco, Basel, Switzerland) supplemented with 0.65 g glucose and 200μM kynurenate at pH 7.4 (dissection GBSS; dGBSS) and cut in 400 μm thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK). The hippocampal region was dissected from individual slices, which were then prepared for organotypic culture using the same procedures as for organotypic cultures of P8 hippocampus.

For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous perfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) saturated with 95% O2 /5% CO2 and viewed using a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany) and analyzed with MetaMorph software (Universal Imaging Corp., West Chester PA).

Immunohistochemistry

Imaged slices were prepared for immuno-staining by microwave-assisted fixation as described (Jensen & Harris, 1989; Roelandse et al., 2003). Briefly, slices were transferred into pre-warmed fixative (4% PFA, 0.5% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer (CCB), pH 7.3) and irradiated at maximum power (1000 W) for 9 sec in a Bio-Rad H2500 microwave processor. Post-irradiation temperature, measured by a built-in temperature probe, was 35 - 50° C. Slices were washed, cryoprotected using 25% sucrose and cut in 12-18µm serial cryosections. The sections were incubated for 30 min in absolute methanol containing 0.3% H2O2 to block endogenous peroxidase activity and subsequently in Tris buffered saline (TBS) with 0.2% Triton X-100 and 10 % normal goat serum (Gibco, Basel, Switzerland) to block non-specific binding of the used antibodies. Sections were then exposed overnight to peroxidase-conjugated isolectin (Sigma St Louis MA, a kind gift of Dr. M. Meins, see Monnet-Tschudi et al., 1996) and either mouse anti-MAP2C (1:5, see Weisshaar et al., 1992) or rabbit anti-GFP (1:800) in 0.3% Triton X-100, 5% normal goat serum (NGS; Gibco, Basel, Switzerland) and 0.1M CCB at room temperature. After washing, sections were incubated correspondingly for 1h at room temperature in goat anti-mouse conjugated alkaline phosphatase (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) or sheep anti-rabbit conjugated alkaline phosphatase (1:80; The Binding Site, Birmingham, United Kingdom) in 0.1M CCB and 5% NGS. Blue staining was developed with 100 mg/ml NBT (2-nitro-blue-tetra-zolium chloride, Roche Diagnostics, Basel Switzerland) and 50 mg/ml BCIP (4-bromo-4cloro-3indolyl-phosphate - Roche Diagnostics, Basel Switzerland) in NTMT buffer (consisting of 100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl2 and 0.1% Triton X-100) and subsequent red staining with an AEC substrate kit (3-amino-9-ethyl-carbazole – Vector Laboratories, Burlingame, CA) with H2O2.

Results

Organotypic cultures have been shown to effectively simulate in vivo neuronal development from various brain regions (e.g. hippocampus: Frotscher, 1992; Roelandse et al., 2003; or spinal cord: Rothstein et al., 1993; Takuma et al., 2002). In the present experiments we used fluorescent and non-fluorescent hippocampal slices from embryonic day 18 (E18) or postnatal day 8 (P8) mice to maintain both homo- and heterochronic co-cultures and studied the dynamics and behaviour of developing axons originating from GFP-expressing neurons.

E18-E18 co-cultures form mature neuronal projections

Since the pattern of expression of the chicken β-actin promoter used to express fluorescent markers in our experiments gives high expression in spine bearing neurons in area CA1 of the hippocampus (Ludin et al., 1996). Consequently, by using GFPtKras as a marker and co-cultures derived from expressing and non-expressing littermates (Fig. 1A, inset), we were able to follow both dendritic development in fluorescent cultures as well as the development of outgrowing fluorescent axons into non-fluorescent cultures (Figs. 1A-C). Imaging slices of E18 hippocampus after 10 DIV (n = 2) showed that both dendrites and axons had highly dynamic growth cones at their tip, as described previously (Dailey & Smith, 1996; Wong & Wong, 2000; Chang & De Camilli, 2001; Roelandse et al., 2003). By this time, labelled axons had rapidly extended from the GFPtKras slice and invaded the neighbouring non-fluorescent slice. Close to the GFP-expressing slice, a dense network of neuro-

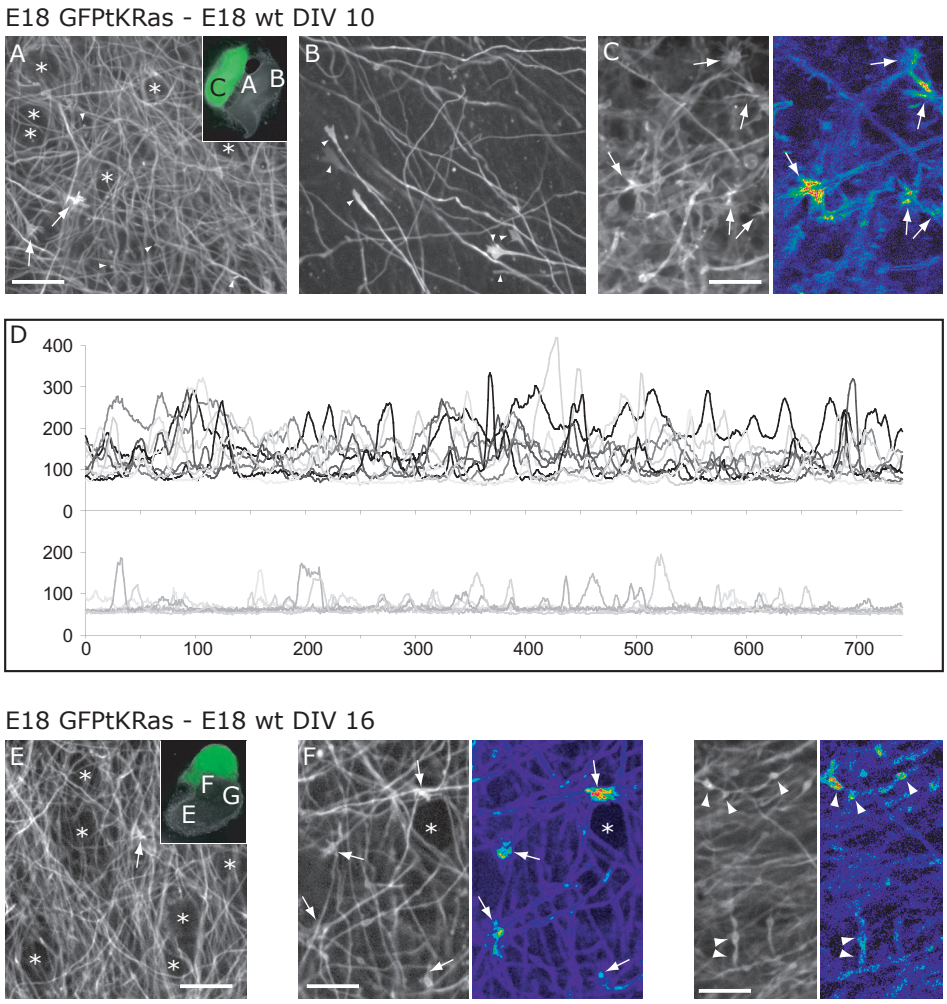


Figure 1: Early neuronal development of E18 GFPtKras – E18 wt co-cultures
(a) The inset shows a low magnification overlay a dark field image and GFP-fluorescence in an E18 GFPtKras – E18 wt co-culture at 10 DIV. A, B and C indicate the approximate location of the corresponding figures. Panel A shows several axons from GFP-expressing neurons invading the non-fluorescent culture. At this early stage of the culture, several growth cones can be found (arrows) as well as filopodia-like protrusions along the axon (arrowheads). The open areas suggest the presence of non-fluorescent cells (asterisks). Bar for (a) and (b) = 8 µm. (b) The leading edge of the axons can be found further ahead in the non-fluorescent culture. Also here, several highly dynamic growth cones can be found (arrowheads). (c) Neurons in area CA1 of the fluorescent culture are very immature and extending many dynamic dendritic protrusions (arrows). The motility image in the right panel shows the relative dynamics of these growth cones, in which highly motile areas are indicated in red/white and background motility is indicated in black/blue. Bar = 8 µm. (d) Quantification of axon densities in the non-fluorescent culture, both in the vicinity of the fluorescent culture, as shown in (a)(top graph) and across the other side as shown in (b)(bottom graph). Indicated are greyscale values along a line (750 pixels long) in 10 images (top) and 6 images (bottom). When the line crosses an axon, greyscale values will increase thereby each peak in the graph indicating an axon. (e) The inset shows a low magnification image of an E18 GFPtKras – E18 wt co-culture at 16 DIV with the approximate location of the corresponding figures. Panel E shows a high-density axonal network further out in the non-fluorescent culture. Growth cones (arrows) can still be found in these cultures, both far out as well as (f) close by the fluorescent culture and show a high degree of ongoing morphological plasticity (right panel). Both in (e) and (f), asterisks indicate the presumed location of cell bodies. Bar (e) = 8 µm; bar (f) = 4 µm. (g) After 16 DIV some varicosities (arrowheads) start to appear along the axonal shaft and are highly dynamic (right panel). Bar = 4 µm.

nal projections had evolved (Fig. 1A) that appeared as long slender structures with an occasional protrusion along its shaft (Fig. 1A, arrowheads). Open areas in-between the axons are likely to be presumed locations of the non-fluorescent cell bodies (Fig. 1A, asterisks). Several axons had advanced across the entire slice, resulting in an area with many growth cones (Fig. 1B). However, the density of axonal projections was much higher in the vicinity of the fluorescent slice (Fig. 1D, top panel) as compared to the density of axons that had advanced across the entire slice (Fig. 1D, bottom panel).

At the same time we could observe the development of dendrites that originated from similar GFP-expressing CA1 pyramidal neurons invading the non-fluorescent slice with their fluorescent axons. These dendrites also had developed numerous growth cones and extended but remained within the slice of origin (Fig. 1C, left panel). These growth cones were highly motile over the course of several minutes, indicated by the red colour in the motility image (Fig. 1C, right panel), suggesting a high degree of morphological plasticity.
In these cultures established at E18, the cultured tissue remained permissive for ingrowing axonal growth

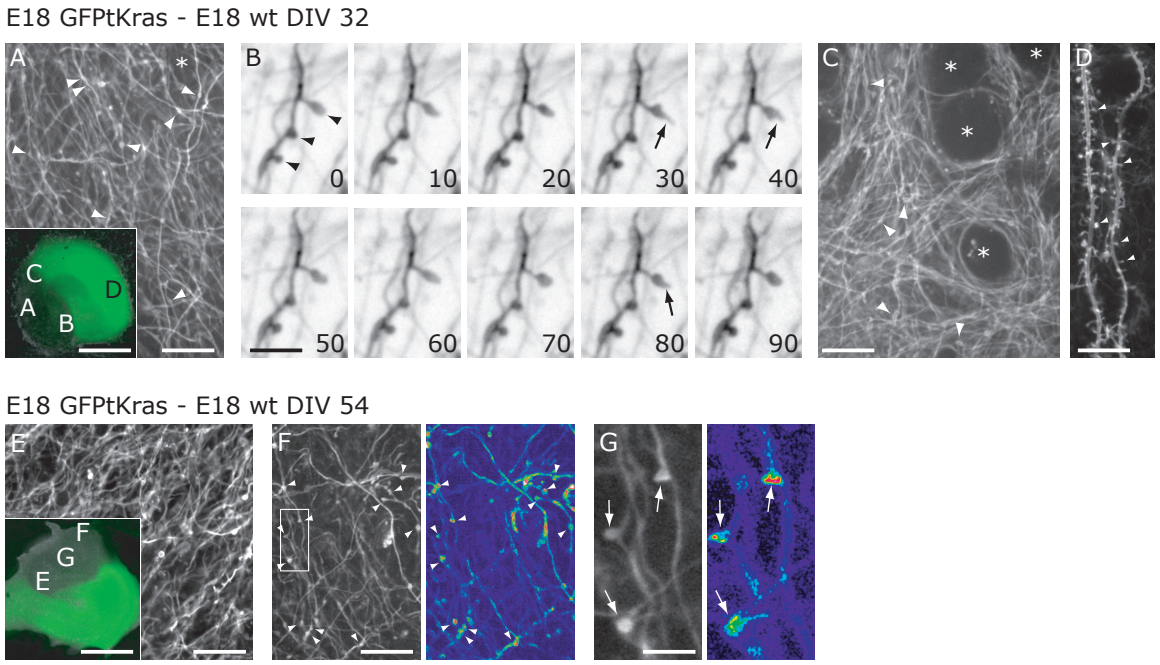


Figure 2: Cross-innervations leads to mature axonal boutons in E18 GFPtKras – E18 wt co-cultures
(a) The inset shows a low magnification image of an E18 GFPtKras – E18 wt co-culture at 32 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel A shows an area in the non-fluorescent culture where numerous axons have established synaptic varicosities (arrowheads). Bar = 8 µm. (b) After 32 DIV, axonal varicosities (arrowheads) still remain motile as can be seen in this series of high magnification images take at 10 sec interval. The contrast has been inverted for clarity. Note the rapid extension and retraction of a protrusion from the head of one of the axon terminals (arrow). Bar = 3 µm. (c) Axons have formed a stable and dense network throughout the non-fluorescent culture with many varicosities along their shaft (arrowheads) closely surrounding cell bodies (asterisks). Bar = 12 µm. (d) Dendrites in the GFP-expressing culture have developed after 32 DIV mature dendritic spines along their shaft. Bar = 12 µm. (e) The inset shows a low magnification image of an E18 GFPtKras – E18 wt co-culture at 54 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel E shows that once axonal varicosities have been established, axons will remain in the non-fluorescent culture, however, (f) from 4 weeks in vitro onwards, morphological plasticity in these cultures remains restricted to synaptic varicosities (arrowheads), as can be seen from the localized motility in the right panel. The boxed area is shown at higher magnification in (g). Bar (e)/(f) = 8 µm. (g) Three axonal varicosities with their corresponding motility index. Note that both axonal shaft and background are relatively deprived of any dynamics. Bar = 2 µm

cones after 16 DIV (n = 3 cultures, Figs. 1E & 1F). An intense network of neuronal projections was now present throughout the non-fluorescent culture (Figs. 1E-G) and the present axonal growth cones were highly dynamic (Fig. 1F). Some of the first formed axons that were in the vicinity of the GFPtKras culture had formed varicosities along their shafts (Fig. 1G, left panel). Varicosities are a key feature of late developing and mature axons (Ishizuka et al., 1990; Gomez-Di Cesare et al., 1997; Shepherd & Harris, 1998) and suggest the formation of presynaptic vesicle release machinery. Similar to their postsynaptic counterparts, these structures changed shape rapidly on a seconds time-scale and were focal points of morphological plasticity as can be seen by comparing background dynamics (indicated in blue) with the dynamics of the varicosities (red, Fig. 1G, left panel).

Previous studies have shown that dendrites mature in organotypic hippocampal cultures over the course of 4 weeks (Frotscher et al., 1988; Roelandse et al., 2003). We therefore examined our cultures after 4 weeks, once dendrites had matured. After 32 DIV the intense network of axonal projections remained present in the non-fluorescent culture and the majority of axons had formed numerous varicosities along their shaft (Fig. 2A). High-resolution time-lapse imaging showed that these varicosities had retained their morphological plasticity (Fig. 2B) with protrusions extending and retracting within several seconds (Fig. 2B arrow) as well as subtle changes in diameter and orientation. Overview images, such as that in Fig. 2C showed a multitude of axons closely surrounding the presumed locations of non-fluorescent cell bodies (Fig. 2C). As expected, dendrites in the GFP-expressing slice had formed mature dendritic spines along their shafts (Fig. 2D) with membrane dynamics now restricted to the spine head (data not shown). After 54 DIV axons were still present in large numbers suggesting that a successful, long-duration cross-innervation had been established between the two cultures (Fig. 2E). After long periods of maturation in vitro (n = 6 cultures), growth cones were absent in either culture and instead axons present throughout the cultures showed abundant varicosities indicative of

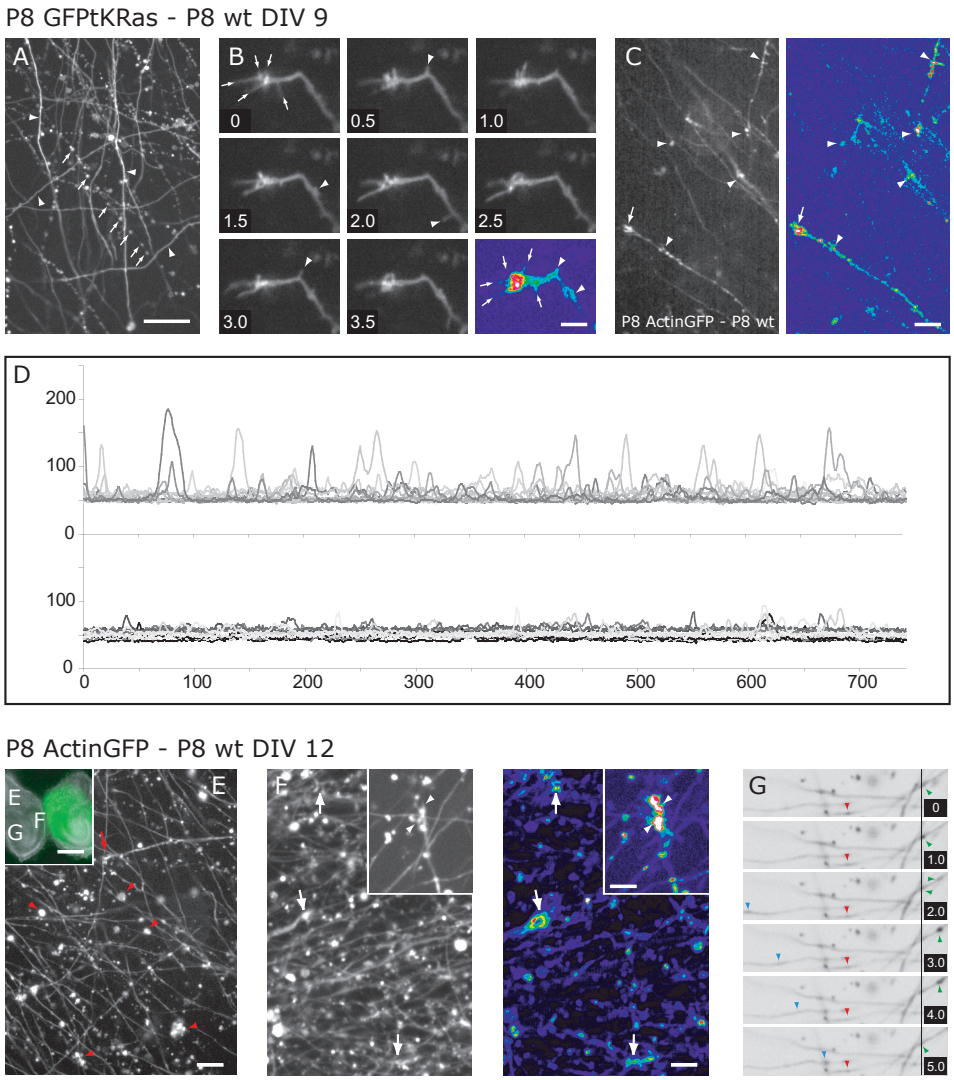


Figure 3: Early neuronal development of P8 GFPtKras – P8 wt co-cultures
(a) Co-culturing P8 GFPtKras – P8 wt results in the outgrowth of GFP-labelled axons in the non-fluorescent neighbouring culture (arrowheads). Nonetheless, many of these axons degrade rapidly after formation. Swollen axons or remnants of fluorescent axons along a line can often been found (arrows). Bar = 8 µm. (b) Areas that are highly motile in these co-cultures co-localize with the presence of actin, as can be seen using actin-GFP as a fluorescent marker for axonal outgrowth in the non-fluorescent culture. By comparing left and right panel, it can be seen that both growth cones (arrow) and protrusions along the axons (arrowheads) are highly motile and contain actin. Bar = 3 µm. (c) Axonal growth cones display rapid morphological plasticity as shown here on images taken 0.5 min apart. Both protrusions extend and retract over the time scale of seconds both at the tip of the growth cone (arrows) as well as along the axonal shaft (arrowheads). The last panel shows a motility image of the same growth cone of a total recording time of 9 min. Bar = 4 µm. (d) Quantification of axon densities in the non-fluorescent culture, both in the vicinity of the fluorescent culture, as shown in (a)(top graph) and across the other side as shown in (b)(bottom graph). Indicated are greyscale values along a line (750 pixels long) in 12 images (top) and 11 images (bottom). When the line crosses an axon, greyscale values will increase thereby each peak in the graph indicating an axon. (e) The inset shows a low magnification image of a P8 GFPtKras – P8 wt co-culture at 12 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel E shows some fluorescent axons with an extending growth cone (arrow) at the far end of the non-fluorescent culture. Arrowheads indicate several accumulations of fluorescent material, either auto-fluorescence or material from degraded fluorescent axons. Bar = 4 µm. (f) Closer to the fluorescent culture, fluorescent axons have formed a dense network with many protruding growth cones (arrows). These are, as compared to axonal shafts or the surrounding tissue, highly dynamic (arrows, right panel). The inset shows a higher magnification of one of the growth cones in the culture, with its corresponding motility image. Bar = 4 µm; bar inset = 2 µm. (g) Using actin-GFP as a marker for axons extending in the non-fluorescent culture, rapid movements of fluorescent 'packages' were observed along the axonal shaft. In this series of images, taken 1 min apart, one package remained stable over the imaged period (red) and another moved fast rapidly along the shaft (blue). The line indicates the starting position of the third package that separated in two packages ("2.0") that subsequently rejoined and disappeared (green).

the presence of mature presynaptic boutons (Figs. 2F-G, left panels). Despite this fundamental stability these varicosities had preserved a high degree of membrane dynamics (Figs. 2F-G, right panels).

Lack of axonal consolidation after initial axonal invasion in P8-P8 co-cultures

Actin has been suggested to be involved in developing presynaptic structures (Luo, 2002) and in mature axonal boutons, to scaffold regulatory proteins (Sankaranarayanan et al., 2003). In addition the shown activity-dependent changes in the number of axonal varicosities (Hatada et al., 2000; Colicos et al., 2001; Luthi et al., 2001; De Paola et al., 2003) are likely to be actin depend. We therefore explored the use of transgenic mice expressing actin-GFP (Fischer et al., 2000) as means to fluorescently label axons in our co-culture setup to further examine

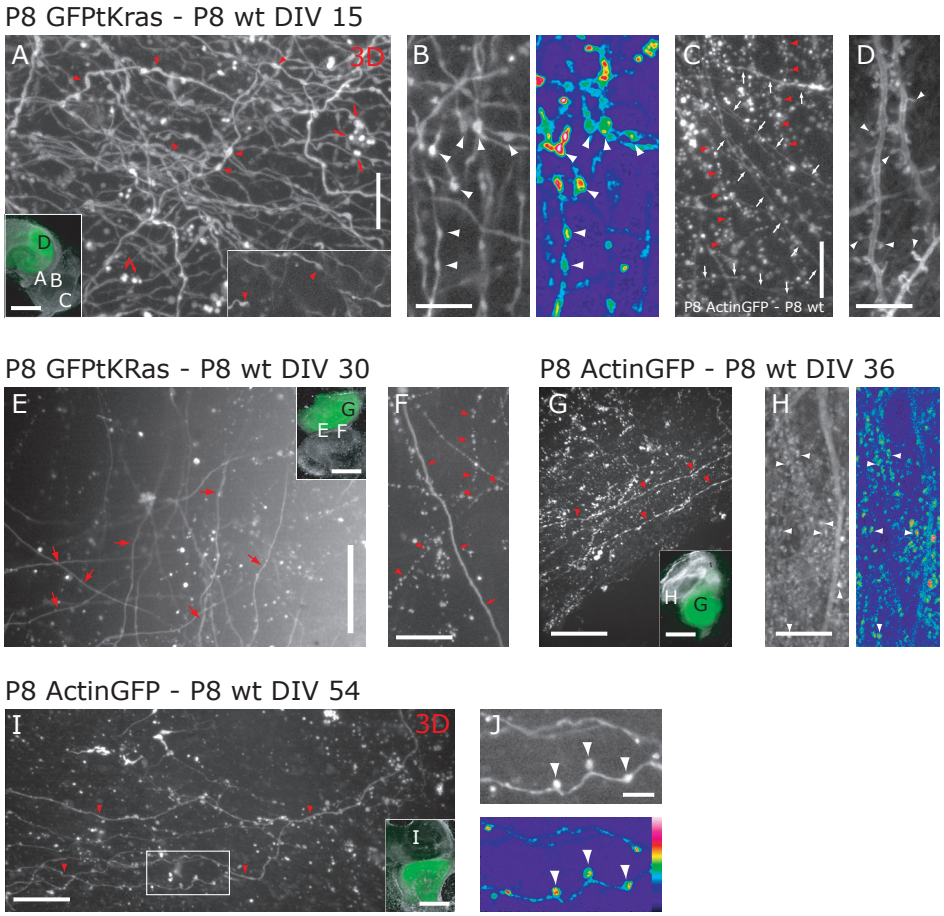


Figure 4: Co-cultures of P8 GFPtKras – P8 wt are unable to maintain synaptic connections
(a) The left inset shows a low magnification image of a P8 GFPtKras – P8 wt co-culture at 15 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel A shows a projection through a z-series of 20 images, taken 0.3 µm. apart. The right inset shows the image of a single plane take from the same series. Despite the fact that several axons have formed a network with mature axonal varicosities (arrowheads) in the non-fluorescent culture, still several accumulations of fluorescent material can be found (arrows). Bar = 8 µm. (b) Morphological plasticity (right panel) on axons is restricted to its varicosities (arrowheads). Bar = 4 µm. (c) Further away from the fluorescent culture, most axons have degenerated leaving residues of fluorescent material behind (arrowheads). Bar = 8 µm. (d) In the fluorescent culture, dendrites start to develop mature dendritic protrusions on the surface (arrowheads). Bar = 8 µm. (e) The inset shows a low magnification image of a P8 GFPtKras – P8 wt co-culture at 30 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel E shows an area next to the fluorescent culture. After 30 DIV, only in the vicinity of the fluorescent culture, axons could sparsely be found (arrows). Bar = 12 µm. (f) Axons were typically straight and often lacked the presence of axonal varicosities (arrows). Frequently, remnants of fluorescent axons were found along a line (arrowhead). Bar = 8 µm. (g) The inset shows a low magnification image of a P8 GFPtKras – P8 wt co-culture at 36 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. Panel E shows an area with 2 axons (arrowheads) devoid of apparent varicosities surrounded by leftovers of fluorescent axons. Bar = 12 µm. (h) Postsynaptic components in the fluorescent culture had developed normally with dendritic spines along the dendrite (arrows) that exposed localized morphological plasticity (right panel). Bar = 8 µm. (i) The inset shows a low magnification image of a P8 GFPtKras – P8 wt co-culture at 54 DIV with the approximate location of the corresponding figures. Bar inset = 500 µm. In rare cases, mature axons (arrowheads) can be found further away in the non-fluorescent culture, which have axonal varicosities along their shaft. The box area is shown in (j). Bar = 12 µm. (j) Rapid actin-based dynamics in the non-fluorescent culture are constrained to axonal varicosities (arrowheads) along GFP-labelled axons. Bar = 3 µm.

the role of actin in presynaptic maturation.

In co-cultures derived from P8 animals, fluorescent axons had invaded the non-fluorescent culture by 9 DIV but were far less numerous than those in E18-E18 co-cultures (n = 3 cultures, Fig. 3A). At this time, the majority of the invading fluorescent axons were still in the vicinity of the GFP-expressing slice (Fig. 3D, top panel) however some axons had advanced across the entire slice (Fig. 3D, bottom panel).

In P8-P8 co-cultures degenerating axons, indicated by intensely swellings along their length, were common (Fig. 3A, arrows). In cultures of actin-GFP expressing slices, both growth cones and filopodia-like protrusions along the axonal shaft contained high amounts of actin (Fig. 3B), consistent with previous reports (Okabe & Hirokawa, 1991; Bradke & Dotti, 1999; Luo, 2002). A closer assessment of GFPtKras labelled axons in the non-fluorescent cultures showed the rapid extension and retraction of both filopodia on a growth cone and protrusions along the axonal shaft (Fig. 3C).

After 12 DIV (Fig. 3E), tissue remained permissive for ingrowing new axons and most had invaded the non-fluorescent cultures up to its outer edge and more fluorescent remnants of axons appeared (n = 4 cultures). By this time, close to the GFP-expressing culture, the axonal network had become denser (Fig. 3F, left panel), to an extent similar to that observed in E18-E18 co-cultures (Fig. 3F, inset). Membrane dynamics along axons were most prominent on their growth cones (Fig. 3F, right panel and inset), but apart from the characteristic presence of actin in both growth cones and filopodia-like protrusions, small clusters of actin-GFP were found in the axon moving within several minutes in both directions along axonal shafts (Fig. 3G). These movements were most prominent in the original time-lapse recordings of the same region.

Between 15 DIV and 30 DIV, axons had extended further in the non-fluorescent culture but at the same time had become less numerous (n = 9 cultures). Figure 4A shows a 3-dimensional projection through a stack of 20 images, taken 0.3 μm apart whereas the bottom right inset illustrates a region of the same projection but instead taken from a single plane. Several varicosities had formed along the axonal shaft (Fig. 4B, left panel) and these co-localized with a high degree of morphological plasticity (Fig. 4B, right panel), similar to that observed for mature axons in E18-E18 co-cultures. Further out in the non-fluorescent culture most fluorescent axons had degenerated and could be observed as a line of fluorescent packages (Fig. 4C). In contrast, dendrites in the fluorescent culture had developed normally, with filopodia-like protrusions and protospines along its shaft (Fig. 4D).

Once the culture had reached its mature state after a month in vitro, as judge by dendritic fine structure, the vast majority of fluorescent axons had disappeared from the non-fluorescent culture (n = 7 cultures). Figure 4E shows a low-magnification image of an area in the very close vicinity of the fluorescent culture, in which the bottom of the panel is aligned with the beginning of the fluorescent culture. As can be noticed, some rare axons could still be found but most of them were uncurled without the obvious presence of varicosities (Fig. 4F). Similar data were obtained using actin-GFP as a fluorescent marker (Fig. 4G) suggesting that the inability of axons to consolidate in the non-fluorescent culture had no relation with the presence of either fluorescent marker. Despite the disappearance of axons from the neighbouring slice, dendrites and dendritic spines in the fluorescent culture had matured normally (Fig. 4H, left panel) with the characteristic focalized morphological plasticity at the tip of their spine head (Fig. 4H, right panel). In one case axons had been able to maintain their varicosities beyond 1 month in culture (Fig. 4I). Interestingly, also here strong morphological plasticity was located at these varicosities (Fig. 4J).

In an attempt to achieve the expected cross-innervation as observed for E18-E18 co-cultures, we prepared P8-P8 co-cultures in various orientations (compare insets Figs. 3E, 4A, 4E, 4G and 4I) and of cerebral cortex (data not shown). Neither variation resulted in a sustained and dense axonal network beyond 15 DIV.

Degeneration of P8 tissue in heterochronic co-cultures of E18-P8 hippocampus

To further investigate the observed difference between E18 and P8 neuronal tissue in ability to form mature axonal circuits with the neighbouring slice, we made heterochronic co-cultures of E18-P8 hippocampus. Using GFP-expressing E18 hippocampus and taking into account the fact that E18 axons had successfully made mature varicosities in the neighbouring slice, we reasoned that if these axons were now unable to consolidate

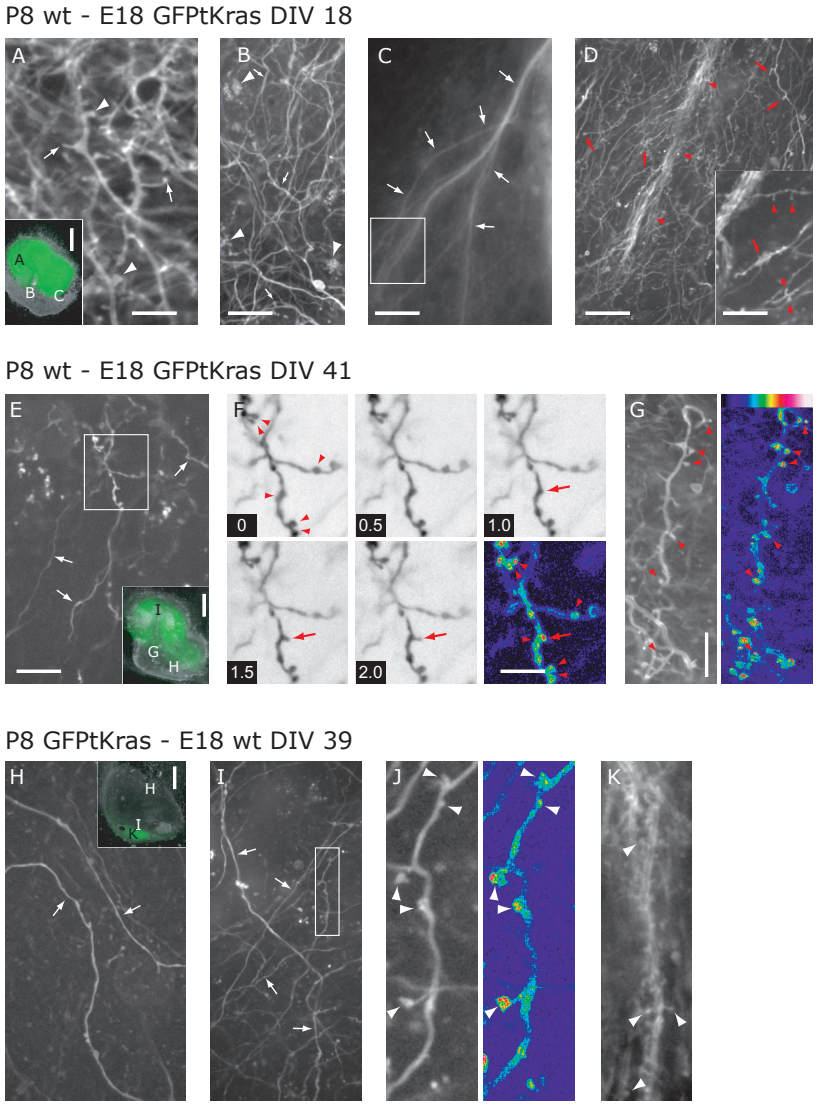


Figure 5: Rapid degeneration of P8 tissue in heterochronic cultures of P8 and E18 hippocampus
(a) The inset shows a low magnification image of an E18 GFPtKras – P8 wt co-culture at 17 DIV with the approximate location of the corresponding figures. Note the shrunken size degenerated appearance of the non-fluorescent P8 slice. Bar inset = 500 µm. Neuronal development of postsynaptic structures in the fluorescent E18 culture is normal, with filopodia-like protrusions and protospines rapidly extending and retracting along the dendrite (arrows) as well as dendritic spines (arrowheads). Bar = 6 µm. (b) GFP-labelled axons (arrows) originating from the E18 slice extend in the non-fluorescent P8 culture. Some fluorescent deposits (arrowheads) can be found among the axons. Bar = 12 µm. (c) Bundle of fluorescent E18 axons (arrows) invading the non-fluorescent P8 cultures. The boxed area is shown in (d) as a confocal image. Bar = 60 µm. (d) Numerous axons (arrowheads) bifurcate in the non-fluorescent culture and extend several dynamic protrusions (arrows) along its shaft. Bar = 14 µm. The inset illustrates a high magnification image of such protrusions (arrowheads) and a growth cone (arrow) in-between the axonal branches. Bar inset = 8 µm. (e) Rare consolidation of E18 axons in P8 tissue. The inset shows a low magnification image of an E18 GFPtKras – P8 wt co-culture at 41 DIV with the approximate location of the corresponding figures. Note the further degeneration of the non-fluorescent P8 slice. Bar inset = 500 µm. Panel E shows a few axons (arrows), originating from the fluorescent E18 neurons, that have invaded the non-fluorescent P8 tissue. The boxed area is shown in (f). Bar = 12 µm. (f) Shown are several planes taken from a time lapse series with images taken 0.5 sec apart. A rapid extending protrusion (arrow) and several mature synaptic varicosities (arrowheads) can be seen along the axonal shaft, which expose a high degree of localized motility (bottom right panel). Bar = 7 µm. (g) In the GFP-labelled E18 culture, dendrites have developed mature dendritic spines (arrowheads) with their characteristic focalized morphological plasticity (right panel). Bar = 7 µm (h) Rare consolidation of P8 axons in E18 tissue. The inset shows a low magnification image of a P8 GFPtKras – E18 wt co-culture at 39 DIV with the approximate location of the corresponding figures. Note the small amount of GFP-labelled P8 tissue is left in the co-culture, in contrast to the non-fluorescent E18 slice. Bar inset = 500 µm. Two axons (arrowheads) originating from P8 neurons expressing GFPtKras have invaded the non-fluorescent E18 tissue till its far end. Bar = 12 µm (i) Closer to the fluorescent P8 slice, some more GFP-labelled axons can be found (arrows). The boxed area is shown in (l). Bar = 12 µm (j) GFP-labelled axons originating from P8 slice that invade the non-fluorescent E18 tissue express high motility at the sides of axonal varicosities (arrowheads, right panel). Bar = 3 µm (k) In the GFP-labelled P8 culture some mature dendrites can be found with dendritic protrusions (arrowheads). Bar = 6 µm.

in a months' time, this would be indicative that postsynaptic P8 neurons were non-permissive for synapse formation. Vice versa, using GFP-expressing P8 hippocampus and taking into account that E18 dendrites are permissive to invading axons from the neighbouring slice, we reasoned that if these P8 axons were still unable to consolidate over a months' time, presynaptic P8 neurons were non-permissive for synapse formation.

These experiments produced a surprising result; after 14 DIV the P8 slice in these heterochronic co-culture had drastically shrunk to a size smaller then its E18 neighbour (n = 10 cultures, Fig. 5A, inset), whereas the size of a P8 slice was larger to start with at 0 DIV. After 18 DIV neurons in E18 cultures were in the middle of neuronal development, showing various protospines and some dendritic spines (Fig. 5A) with their characteristic motility (data not shown). GFPtKras-labelled axons had invaded the non-fluorescent P8 culture but at the same

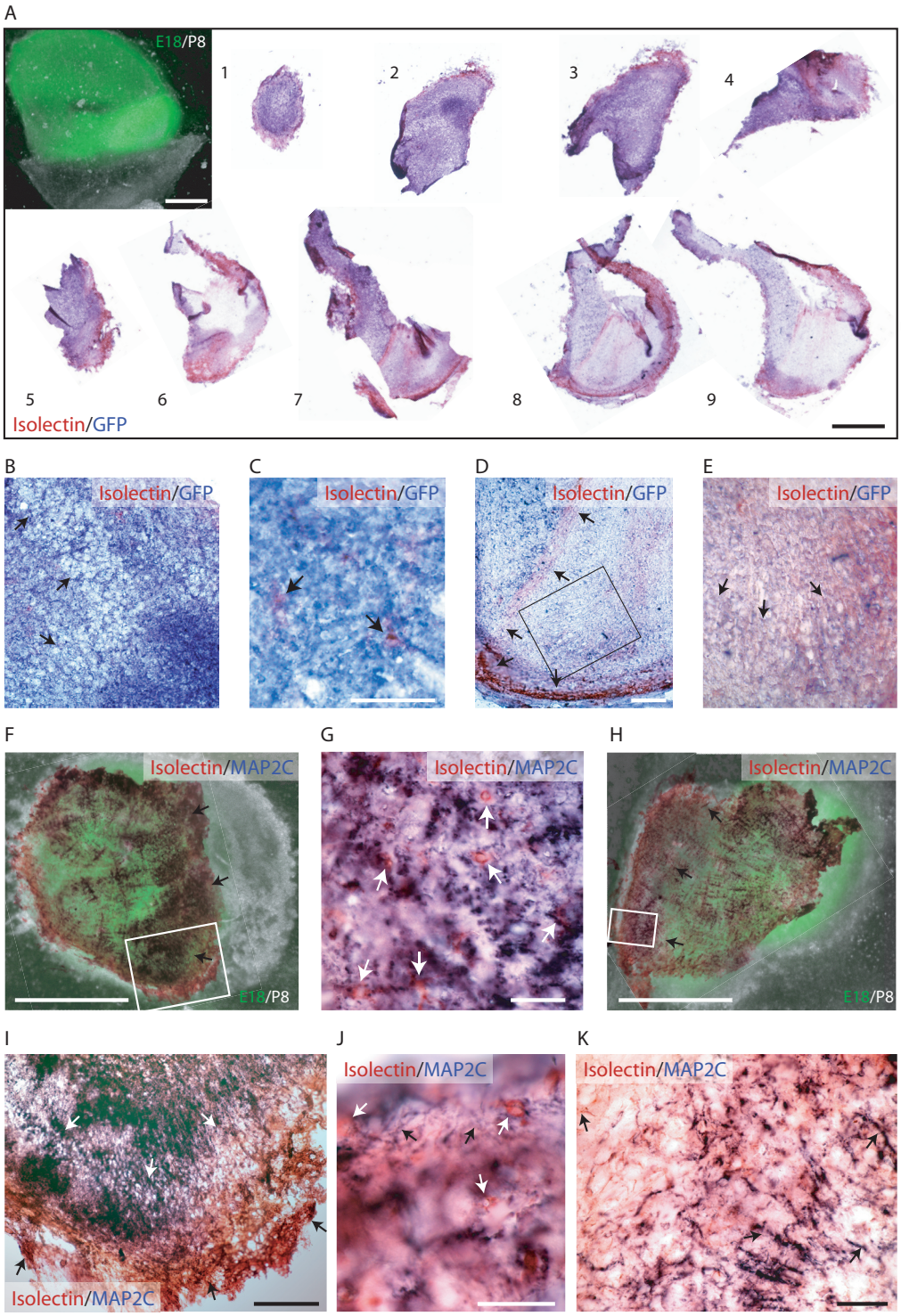


Figure 6: Microglia proliferation in P8 tissue in heterochronic cultures of P8 and E18 hippocampus
(a) A low magnification overlay image of dark field and GFP-fluorescence of an E18 GFPtKras – P8 wt co-culture at 41 DIV. Subsequent images labelled 1-9 show serial cryosections through the same culture and stained for GFP (blue) and isolectin, a marker for microglia (red). The sections are oriented in the same way as the fluorescent image (a). A thick layer of microglia surrounds the right side of the co-culture, representing the degenerated P8 slice. Bar = 0.5 mm (b) Higher magnification of the pyramidal cell layer (arrows), as shown in (a2), in the E18 GFPtKras slice. Note the density of the neurons even after 41 DIV suggesting these neurons not to be affected by the neighbouring degenerating slice. Bar = 0.1 mm (c) E18 slices contain some microglia (arrows) at low density in the neuropil. Image taken from (a1). Bar = 0.1 mm (d) Higher magnification of (a8) showing the difference in invasion of microglia between the E18 slice (left top) and the P8 slice (right). The boxed area surrounding a part of the P8 slice is shown at higher magnification in (e) Bar = 0.1 mm (e) Absence of GFP staining in the non-fluorescent P8 slice. Note the spare amount of presumed pyramidal cells (arrows). Bar = 0.1 mm (f) Haematoxylin-Eosin staining on a single hippocampal slice culture at 41 DIV (top panel) showing a good preservation of organ-typical structures as CA1 and dentate gyrus (DG) as well as a clear staining of nuclei in the pyramidal cell layer in CA1 (bottom panel). Bar top = 1 mm (g) Overlay image of both dark field and GFP-fluorescence taken of the entire culture (green/white) as well as one stained serial section trough parts of the culture (blue/red). The arrows mark the border between the non-fluorescent P8 slice and the fluorescent E18 slice. The P8 slice is not present in this section and hence not stained. Note the red staining surrounding both cultures. The boxed area is shown in (j). Bar = 1 mm (h) High magnification image of the neuropil in the E18 slice. Several microglia (red, arrows) can be observed in the molecular layer of area CA1. Bar = 0.05 mm (i) Another overlay image of both dark field and GFP-fluorescence taken of the entire culture (green/white) and in addition one stained serial section trough parts of the culture (blue/red). The arrows mark the border between the non-fluorescent P8 slice and the fluorescent E18 slice. Some neurons could still be found in the P8 culture but in low density. The boxed area is shown in (l). Bar = 1 mm (j) Densely packed pyramidal cell layer of the E18 slice (blue; white arrows) surrounded by a layer of microglia (red; black arrows). Note the microglia layer to increase in thickness towards the P8 slice (top right). Bar = 0.5 mm (k) High magnification image of the neuropil in the P8 slice. Several microglia (red; arrows) can be observed in the molecular layer of area CA1 as well as several MAP2C-positive dendrites (blue; black arrows). Note the difference in neuronal and microglial density between (h) and (k). Bar = 0.05 mm (l) Higher magnification of the pyramidal cell layer of the P8 slice with some dendritic projections (arrows) to be observed as well as a thick layer of microglia. Bar = 0.5 mm

time various clusters of (auto) fluorescent material were present (Fig. 5B) suggestive of degenerating tissue. Bundles of axons were found close to the GFP-expressing E18 slice invading the non-fluorescent P8 tissue (Fig. 5C). Higher magnification showed multiple branching axons (Fig. 5D) that were motile in time-lapse recordings (data not shown) and exhibited several characteristic features of developing axons, such as filopodia-like protrusions along the axonal shaft and growth cones (Fig. 5D and inset).

After 41 DIV, once neurons had fully matured, E18 fluorescent axons were sparse in the non-fluorescent P8 culture (n = 8 cultures, Fig. 5E). However, these had consolidated and formed varicosities along their shaft that changed shape rapidly over several minutes (Fig. 5F), indicative of synaptic connections between E18 and P8 neurons. The majority of the axonal projections were found in the vicinity of the E18 slice. Within the E18 culture, dendrites had matured normally with various dendritic spines (Fig. 5G). The degeneration of the P8 culture was most evident in GFP-expressing slices (Fig. 5H, inset). Nonetheless, axons could be found in the non-fluorescent neighbouring E18 slice, either far off (Fig. 5H) or in the vicinity of the GFPtKras-expressing slice (Fig. 5I). Most axons were uncurled and lacked the presence of regularly spaced varicosities along their shafts. Even so, those rare varicosities found were highly dynamic (Fig. 5J). As could be expected by the above shown presence of P8 GFPtKras-labelled axons in the non-fluorescent E18 culture, we found some dendrites in the GFP-expressing culture (Fig. 5K).

Proliferating microglia surround P8 tissue in heterochronic co-cultures

A possible reason for the shrinkage of P8 tissue in heterochronic cultures might be the activation of microglial cells known to be involved in lesion-induced degradation of neurons (Thanos & Mey, 1995; Eyupoglu et al., 2003b). To further investigate this possibility, we performed histochemistry on cryosections from the same cultures that had been recorded live (e.g. Fig. 5F, inset) using horseradish peroxidase conjugated Griffonia simplicifolia isolectin B4 (GSI-B4, Monnet-Tschudi et al., 1996) and immunochemistry using primary antibodies against either GFP or MAP2C in combination with alkaline phosphatase conjugated secondary antibodies. Using cryosections of cultures instead of whole-mount staining is most useful for examining overall effects within the organotypic cultures and it provides a good overview of its state (Fig. 6A). Serial cryosections through a heterochronic culture revealed the presence of numerous microglia in these cultures (red staining, Fig. 6A, 1-9). These were prominent in P8 slices forming layer cells thick surrounding the cultured slice (e.g. Fig. 6a: 7) but were less pronounced on E18 slices (e.g. Fig. 6A: 1). In agreement with the data obtained in live cell imaging experiments, we found a thick layer of apparently healthy pyramidal neurons in the E18 slice (Fig. 6B). These were noticeable in the GFP-staining by their non-stained cytoplasm due to the GFP-bound membrane marker. Among the dendritic projections in the molecular layer of are CA1 a sparse distribution of inactive microglia could be found (Fig. 6C). In addition to surrounding the culture, microglia also had formed a thin layer in-between both cultures (Fig. 6D). Within P8 tissue, very few microglia could be found and its molecular layer appeared thinned out (Fig. 6E).

In order to assess the relative proportions of neurons in both slices we examined cryosections of cultures, stained for MAP2C and isolectin, in combination with the previously acquired images from live cell imaging experiments to distinguish P8 from E18 tissue (Figs. 6F and 6H). This illustrated that E18 slices had a thick layer of pyramidal neurons with many protruding MAP2C positive dendrites (Fig. 6F) whereas most neurons in the

Table 1. Time course of axonal development, network density and neuronal degeneration of homo- and heterochronic cultures of E18 and P8 hippocampus

P8 - P8	5 - 10	11 - 15	16 - 19	20 - 30	31 - ...
N =	3	4	4	5	7
Growth cones	X	X	X	-	-
Varicosities	-	-	X	X	X
Dense axonal network	3	4	-	-	-
Some axons	-	-	4	5	4
No axons	-	-	-	-	3
E18 – E18	5 - 10	11 - 15	16 - 19	20 - 30	31 - ...
N =	2	0	1	2	6
Growth cones	X		X	-	-
Varicosities	-		X	X	X
Dense axonal network	2		1	2	6
Some axons	-		-	-	-
No axons	-		-	-	-
E18 - P8	5 - 10	11 - 15	16 - 19	20 - 30	31 - ...
N =	0	10	1	1	8
Growth cones		X	X	-	-
Varicosities		-	X	X	X
Dense axonal network		-	-	-	-
Some axons		10	1	1	8
No axons		-	-	-	-
Degraded P8 tissue		10	1	1	8

The table shows in the top rows the numbers of cultures observed at the different time points, underneath the state of axonal development and in the three bottom rows the average density of axonal networks. Added in the last panel also indicates the relative size of the P8 culture as compared to its neighbouring E18 culture. In all cases, after 10 DIV it had shrunken to a size smaller then the E18 slice.

P8 slice had disappeared as could be observed by the sparse distribution of dendrites against a predominantly red background of staining (Fig. 6I). At a higher magnification, it was noticeable that some microglia had intermingled in the E18 molecular layer of CA1 (Fig. 6G) and similarly in P8 molecular layer (Fig. 6J) however both staining intensities differed visibly (compare Figs. 6G and 6J), suggesting a more prominent presence of microglia together with less dendritic projections in P8 tissue.

Discussion

Research in the past few years has shed much light on postsynaptic development and fundamental behaviour of dendritic spines, from high resolution imaging of Ca2+-fluxes in individual spines (Oertner et al., 2002) and trafficking of receptor subtypes laterally (Borgdorff & Choquet, 2002) as well as by internalization and surface expression (Shi et al., 1999; Inoue & Okabe, 2003; Sans et al., 2003) to the ongoing unravelling of the identity of proteins involved in the postsynaptic density (Sheng & Kim, 2002). In contrast, much less is known about the formation and ensuing fundamental behaviour of axonal boutons. Most studies have focused on axonal path finding, both in dissociate cell cultures (reviewed by Song & Poo, 2001) and in organotypic cultures or in vivo (reviewed by Lopez-Bendito & Molnar, 2003), or on the composition of the presynaptic vesicle release machinery (reviewed by Zamorano & Garner, 2001; Rizo & Sudhof, 2002). Our results confirm previous studies on the axonal outgrowth but are distinct in several ways.

Early postnatal neurons possess an intrinsic capability to for axonal projections

Most past studies have quantified the success of outgrowth in terms of the ability to form axonal projections over the course of several days (e.g. Bolz et al., 1990; Molnar & Blakemore, 1991; Woodhams et al., 1993). Our data suggest that this measure might be misleading since using living co-cultures of early postnatal tissue we

found that despite the rapid and successful formation of axonal extensions in the neighbouring slice during the first two weeks in culture, the vast majority of neurons were unable to preserve these projections over the following weeks. In addition, our data on P8-P8 co-cultures are in contrast with previous studies showing that early postnatal tissue is non-permissive for reciprocal innervation thus suggesting an age-dependent decline in the ability to form axonal projections (Woodhams et al., 1993; Stoppini et al., 1997; Prang et al., 2001). Instead our data suggest an intrinsic capability of early postnatal neurons to sprout after transection, a situation likely to happen during the procedures for hippocampal slice cultures, and suggested previously (Chuckowree & Vickers, 2003). This hypothesis is further supported by the observation that neurons in single hippocampus slice cultures also extend axonal projections in the surrounding plasma clot in the first week in culture (data not shown).

Neurons gradually loose the ability to make new synaptic connections during postnatal development

The observed discrepancy between flourishing cross-innervation of E18-E18 co-cultures and deterioration of most axons in P8-P8 co-cultures may also be attributed to developmental changes between E18 and P8 tissue resulting in the inability of mature CNS to regenerate (Chen & Tonegawa, 1998; Horner & Gage, 2000). At the same time this difference suggests, instead of failing axonal outgrowth, a gradually lost ability to form synapses despite the abundant presence of axonal growth cones and varicosities. Alternatively, in contrast to previous reports suggesting a high correlation between the typical varicosities along axonal shafts and the presence of synaptic release machinery (Shepherd & Harris, 1998), our data suggest these varicosities to be intermediates in axonal maturation formed at random along developing axons (Shepherd et al., 2002). A further examination of the co-localisation of proteins present at the active zone with axonal varicosities together with morphological analysis of electron micrographs taken in our developing co-cultures stained using a horseradish peroxidase product for GFP may further prove this hypothesis. If it were true that some varicosities are intermediates in axonal bouton development, then several varicosities during the first two weeks in culture may not co-localize with active zone proteins or may not be juxtaposed to postsynaptic structures.

A similar temporary increase in the number of varicosities has been observed previously, along Schaffer collaterals leading to CA1, and these differences were most prominent in the second and third week in culture (Gomez-Di Cesare et al., 1997). Like their postsynaptic counterparts, some of these varicosities may become excessive and consequently lost, possibly due to lack of consolidating signals suggested to be released after correlated activity (Weiss, 1941). Images of dendrites in the neighbouring culture revealed the presence of many protrusions and spines along its shaft thereby suggesting enough postsynaptic partners to be present to for new synapses with. However, it cannot be excluded that postsynaptic neurons already acquired sufficient postsynaptic connections once cross-innervating axons arrive (Raisman & Field, 1990) and hence do not form new synapses.

A possible involvement of actin dynamics in developing axons

Previous studies have shown a rapid turnover rate of postsynaptic densities particularly on developing dendrites (Marrs et al., 2001; Grutzendler et al., 2002). Similar to the continuous remodelling of postsynaptic

protrusions along dendrites, our data on P8-P8 co-cultures suggests that the axonal varicosities present early in culture are disassembled at a later stage, implying an ongoing turnover of axonal varicosities in early postnatal development and hence suggest a similar dynamic behaviour for developing presynaptic components. Added to these morphological dynamics of axons at relatively long time scales, we found that mature axons retain this ability but, in contrast to early development, motility was now focalized to the varicosities and appeared at a second's timescale (Figure 2B). Previous studies have shown the presence of actin in presynaptic boutons and attributed its function to scaffolding regulatory proteins in the nerve terminal (Sankaranarayanan et al., 2003) and to regulate synaptic vesicle fusion (Morales et al., 2000). Furthermore the formation of new varicosities upon synaptic facilitation on immature axons is thought to be dependent on actin (Hatada et al., 2000; Colicos et al., 2001). However, neither study has suggested the actin present in presynaptic terminals to serve as a generator of membrane dynamics, as observed in our live cell recordings.

Regarding dendritic spine dynamics, we found both changes in size and diameter of the bouton and rapid extension and retraction of protrusions from the top of the varicosity (see Figs. 2B and 4F). Despite this, we cannot rule out the possibility that most of the observed presynaptic membrane dynamics are passively transferred through the rigid binding between pre- and postsynapse and hence due to the rapid shape changes of their postsynaptic counterparts that have long been known to contain high amounts of actin (Matus et al., 1982). In addition, particularly during development, we found local concentrations of actin-GFP rapidly moving up and down the axonal shaft. Previous work has attributed such local accumulations of actin filaments to predict future axon branching points, locations where both microtubule and actin filament dynamics are essential (Dent & Kalil, 2001). These confined concentrations of actin-GFP may also be involved in fast axonal transport of vesicles transferring plasma membrane and synaptic vesicle proteins to their target (Nakata et al., 1998) as actin itself is thought to travel as monomers via slow axonal transport, in possible association with other actin binding proteins (Mills et al., 1996; Nixon, 1998).

Potential role for microglia invasion in the gradual degeneration of P8 neurons in heterochronic cultures

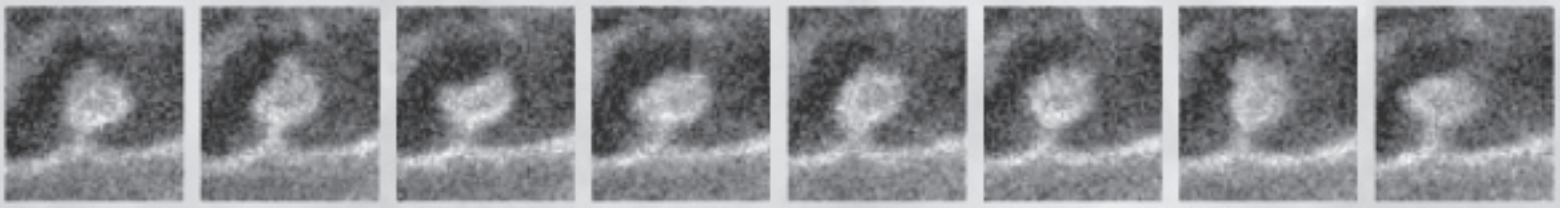
The possibility of co-culturing tissue from different ages has been shown previously (e.g. Woodhams et al., 1993; Stoppini et al., 1997; Zhou et al., 1998; Molnar & Blakemore, 1999) though deterioration of postnatal tissue as observed in our heterochronic co-cultures has not been reported. A possible explanation for this discrepancy may lie in either the origin of the tissue or in the length of time the co-cultures were maintained. The reason for P8 neurons to gradually degenerate in co-cultures of E18 and P8 hippocampus over the time course of several weeks still remains to be seen. One possibility lies in the difference in both expression pattern and function of signaling molecules, resulting in miscommunication between neurons or other cell types of different ages. One candidate protein for causing such miscommunication is the $p75^{NTR}$ neurotrophin receptor, a member of the tumor necrosis factor superfamily that binds all neurotrophins (Dechant & Barde, 2002). Early in development $p75^{NTR}$ is thought to be involved in the modulation of axonal outgrowth (Yamashita et al., 1999; Wong et al., 2002) whereas its expression in adult brain is rigidly upregulated in axotomized neurons within several days post-lesion (Giehl et al., 2001). Moreover, its upregulation has been shown to be involved in the induction of programmed cell death via its binding to nerve growth factor (NGF - Brann et al., 2002; Troy et al., 2002). In E18-P8 co-cultures, a possible source of NGF might be the ingrowing E18 axonal projections that release

neurotrophins. Previous studies have shown that presynaptic cells are capable of releasing neurotrophins at synapses thereby modulating postsynaptic differentiation and cell survival (Haubensak et al., 1998; Heerssen & Segal, 2002). Presynaptically released NFG bound to the upregulated $p75^{NTR}$ might be sufficient to trigger apoptosis in the P8 neurons.

Alternatively NGF might be microglia-derived. Microglia, the resident macrophages of the CNS, are capable of killing cells during early development, a process widely occurring throughout the developing brain. These dying cells all express the $p75^{NTR}$ receptor and are eliminated via cytotoxic mechanisms that involve NFG expressed by microglia as a killing agent (Frade & Barde, 1998). E18 microglia that had migrated to the P8 culture may therefore be responsible for the widespread neurodegeneration of the P8 neurons that are likely to express $p75^{NTR}$. Our microglia staining of the heterochronic cultures are consistent with this hypothesis.
Past studies have shown that lesion-induced degradation of retinal ganglion cells (Thanos & Mey, 1995) and other neurons, including those of the hippocampus (Eyupoglu et al., 2003a) could be delayed by neutralizing microglial activation suggesting a prominent role for microglia in axotomy-induced neuronal death as well as in various neurodegenerative diseases such as Alzheimer (Jellinger & Stadelmann, 2000; Bamberger & Landreth, 2002; Schenk & Yednock, 2002; Van Everbroeck et al., 2002).

An alternative explanation for the observed neuronal degradation of P8 neurons in heterochronic co-cultures might be the difference synaptic or electrical activity that would favour E18 axons making synapses onto P8 dendrites and thereby out-competing P8 neurons. In this scenario, retrograde signals subsequent force P8 neurons to undergo programmed cell-death. It is likely that electrical activities between the two differentially aged slices differ greatly. Embryonic tissue predominantly exhibits spontaneously active independent waves of correlated firing of large populations of neurons that are thought to refine neural circuits whereas postnatal tissue is in the midst of activity-dependent plasticity and refinement that is thought to be instructive and may underlie reorganization of neural circuits (Zhang & Poo, 2001).

HYPOTHERMIA–ASSOCIATED LOSS OF DENDRITIC SPINES



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Abstract

Evidence suggests that hypothermia is associated with regressive changes in dendrite morphology and function (Boycott, 1982; Popov et al., 1992; Aihara et al., 2001; Pyott & Rosenmund, 2002) but the underlying cellular mechanisms are unknown. We investigated the influence of reduced temperature on the morphology and plasticity of dendrites using time-lapse recordings from living tissue slices from transgenic mouse hippocampus expressing a GFP-based neuronal surface marker and electron microscopy of adult brain slices. We find that dendritic spines, the sites of excitatory contacts, are highly sensitive to reduced temperature with rapid loss of actin-based motility followed by disappearance of the entire spine structure at longer times. Our data support previous data suggesting a link between dendritic spine structure and global brain function.

Key Words: synaptic plasticity, hippocampus, time-lapse microscopy, green fluorescent protein, hibernation, hypothermia

Introduction

Dendritic spines are believed to be involved in the modulation of synaptic connectivity underlying learning and memory (Crick, 1982; Matus, 2000), but the relationship between morphological plasticity and cognitive function remains unknown. One clue comes from the study of hibernating animals such as marmots and ground squirrels where it has long been known that the reduction in body temperature and attenuation of awareness is accompanied by loss of dendritic spines (Querton, 1898; Boycott, 1982; Popov et al., 1992). This effect is reversible within 2 hours after re-awakening (Popov et al., 1992) and may be associated with impairment of stored memories (Millesi et al., 2001), suggesting further a link between maintenance of spine morphology and cognitive function. A striking feature of hibernation is the extremely low body temperature, between 0˚C and 8˚C (Hut et al., 2002) of the torpid animal, which is associated with decreased metabolic rate and neuronal activity (Derij & Shtark, 1985; Krilowicz et al., 1989; Igelmund & Heinemann, 1995). However, effects of hypothermia on neuronal function are not limited to hibernating animals; in humans, decreased brain temperature is associated with suppression of bursting activity and electro-cerebral silence (Stecker et al., 2001) suggesting an association between brain temperature and the state of consciousness (Mathew et al., 2001). Hypothermia is commonly used during surgery to protect neurons from ischemia (Sweeney et al., 1985; Griepp & Griepp, 1992; Yu et al., 2000; Bachet & Guilmet, 2002) and is associated with a decreased requirement for volatile anaesthetics to maintain the anesthetized state (Antognini, 1993; Liu et al., 2001). Altogether, these observations suggest a link between hypothermia and plastic changes in dendritic structure. To investigate this potential relationship we took advantage of transgenic mice expressing a GFP-linked neuronal surface marker (Roelandse et al., 2003) to follow the consequences of temperature reduction on dendritic fine structure in living hippocampal neurons.

Methods

Slice preparation and microscopy

Organotypic slice cultures from P8 hippocampus were prepared from transgenic animals expressing GFP-tagged to C-terminal plasma membrane targeting domain of K–ras4B as described (GFPtKras, Roelandse et al., 2003). For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous perfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) saturated with 95% O2 /5% CO2 using a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany) and analyzed with MetaMorph software (Universal Imaging Corp., West Chester PA).

For electron microscopy analysis, hippocampi from mature wild-type mice (n = 3, all 5 weeks old) were dissected in ice-cold, oxygenated buffer (234mM NaHCO3, 1mM NaH2PO4, 8mM MgSO4 and 10mM glucose, pH 7.4) and sliced at 400 µm using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK). Slices were incubated on 0.4 µm porous culture plate inserts (Millipore Corp. Bedford) for 60 min at room temperature in ACSF (117mM NaCl, 5.3mM KCl, 26mM NaHCO3, 1mM NaH2PO4, 1.5mM MgSO4, 2.5mM CaCl2 and

10mM glucose, pH 7.4, equilibrated with 95% O2/5% CO2) with Ca2+ removed to avoid excitotoxic damage (Feig & Lipton, 1990). Subsequently incubation was continued at either 37°C or 4°C for 9h in ACSF saturated with 95% O2 /5% CO2 after which slices were prepared for DiI labelling or electron microscopy by microwave-assisted fixation (Jensen & Harris, 1989). Briefly, slices were transferred into pre-warmed fixative (2% formaldehyde, 6% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer (CCB) pH 7.3) and irradiated at maximum power (1000 W) for 9 sec in a Bio-Rad H2500 microwave processor. Post-irradiation temperature, measured using a built-in temperature probe situated in the fixation solution, was 35 - 50° C. For DiO labelling slices were embedded in 3% agarose, cut transversely at 400 μm using a Leica VT1000s microtome (Leica, Glattbrugg, Switzerland) and shot with DiO coated tungsten particles (0.7 μm diameter) using a Gene Gun (Bio-Rad Laboratories, Hercules, CA) as described earlier (Gan et al., 2000). For electron microscopy, fixed slices were washed in 0.1M CCB, post-fixed with 1% osmium tetroxide and 1.5% potassium ferrocyanide in 0.1M CCB, pH 7.3 and stained with 4% ethanolic uranyl acetate. Serial ultra-thin sections of area CA1 were stained for 30 min with 6% aqueous uranyl acetate then for 15 min with 0.4% lead citrate in 0.04M NaOH.

Results

We examined the influence of reduced temperature on dendritic spines using hippocampal slices from transgenic mice expressing a membrane targeted GFP construct (GFPtKras) which enables the fine structure of dendrites to be recorded in living slices of brain tissue (Roelandse et al., 2003). Observations were made using both acutely cut slices from adult animals and cultured hippocampal slices maintained in vitro for >4 weeks when mature dendritic spines have been established (Frotscher et al., 1988; Roelandse et al., 2003). Cultured slices maintained at 37˚C show stable dendritic spines of mature morphology, which retain actin-based motility at the tips of spine heads (Roelandse et al., 2003). When the temperature was reduced to 24˚C, spines initially retained the same motility as at 37˚C (Fig. 1A, "0"), but subsequently, motility gradually decreased (Fig. 1A, "2": 2 hours) and ultimately stopped completely (Fig. 1A, "4": 4 hours). When incubation at 24˚C was continued overnight, dendrites showed a striking structural regression so that the majority of spines had disappeared (Fig. 1B, right "ON 24˚C"). By contrast, dendrites in cultured slices that had been maintained at 37˚C showed no decline in motility or basic spine morphology even after 8 hours of continuous observation (Fig. 1B, "0" and "8").

Re-warming "cooled" cultures to 37˚C resulted in immediate and rapid outgrowth from the surface of dendrites of highly motile protrusions (Fig. 1C) similar in appearance to spine precursors on developing dendrites (Dailey & Smith, 1996; Roelandse et al., 2003). Quantifying the number of spines per 100 pixels of dendrite length in single planes from confocal images showed that the number of protrusions classified as spines dropped to approximately 1/3 of the control value after incubation overnight at 24˚C (Fig. 1D; $p<0.05$, n=73 dendrites at 37˚C; 100 dendrites at 24˚C). Spine recovery was remarkably effective so that spine density had returned to control levels 24h after rewarming to 37˚C (Fig. 1D; $p<0.05$, n=23 dendrites). Cooling slices to lower temperatures still showed even more severe effects so that a brief exposure of cultures to 4˚C for 30 min was sufficient to freeze spine motility (Fig. 1E).

To check that these effects were not limited to slices in culture or were an artefact of expressing the GFPtKras transgene, we examined acute cut slices from adult hippocampus in which dendrite structure was visualized

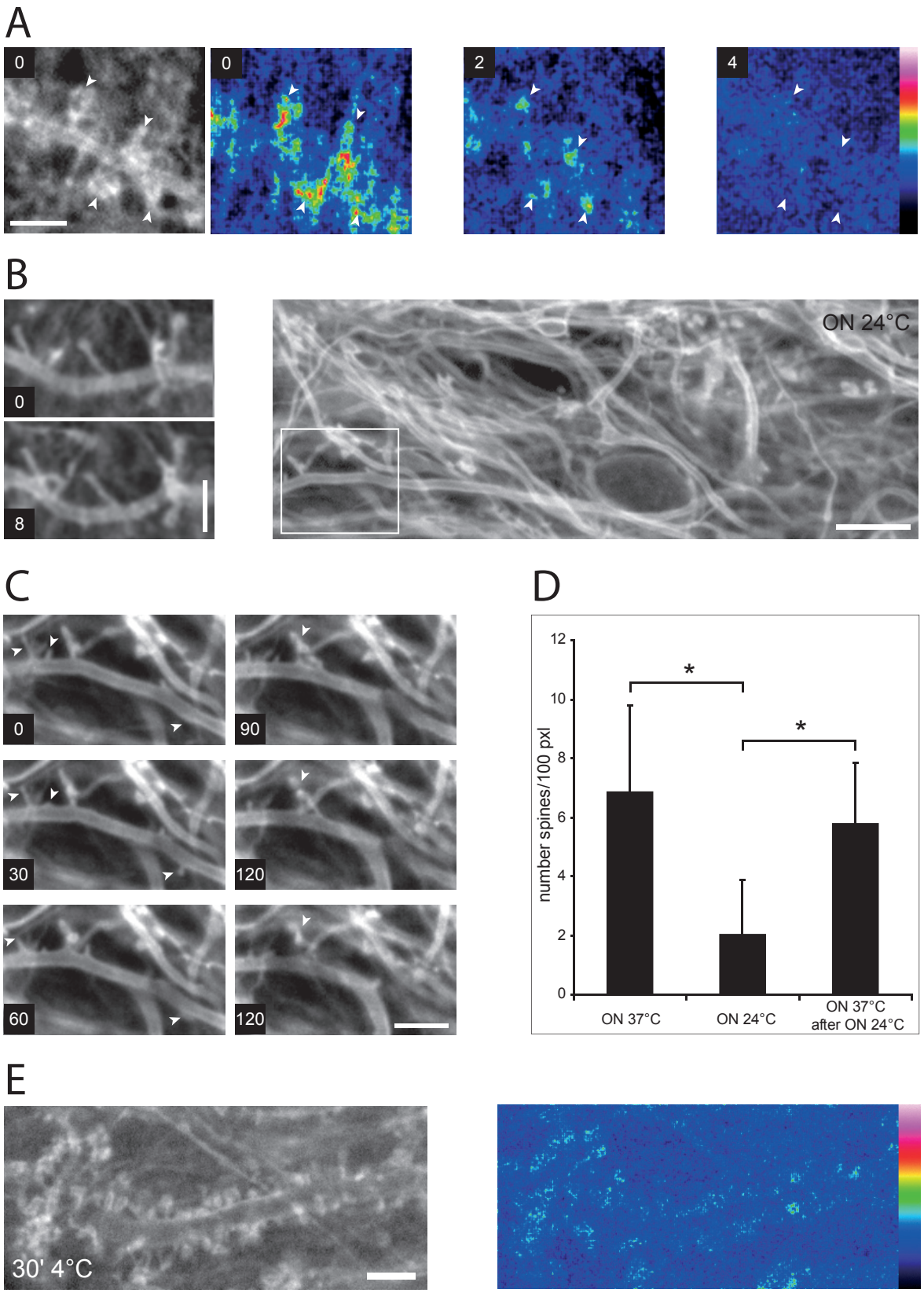


Figure 1: Culturing mature brain slices at room temperature (24°C) seizes spine motility and leads to the disappearance of the majority of spines
(a) Top left shows one frame from a time series with two dendrites in a mature hippocampal slice culture. Right three panels ("0", "2" and "4") show motility images of the corresponding time series with areas of high motility shown in red/white and background motility in blue/black. Images were acquired every 15 sec. Initially, when the cultures are kept at 24°C, spines are still motile (left, 0 hours), however, over time spine motility disappeared (right, "4"). Bar = 3 µm (b) Left two images show a dendrite maintained at 37°C at time "0" and 8 hours later ("8"). Right panel shows several dendrites in a mature slice culture that had been incubated overnight at 24°C and the majority of spines have disappeared from dendrite surface. The boxed area is shown in (c). Bar left panel = 3 µm; bar right panel = 10 µm (c) Subsequent re-warming of the same 'cooled' slice to 37°C induces motile protrusions (arrowheads) to re-emerge from dendrite surface. Images are taken 30 sec apart. Bar = 5 µm (d) Quantification of the number of spines per 100 pixels dendrite length. Overnight cooling results in a significant but reversible reduction in the number of spines (e) Brief exposure (30 min) of mature slice culture to 4C° stops spine motility. Dendrites were imaged at 24°C and the corresponding motility image is shown on the right. Note the small spines. Bar = 3 µm

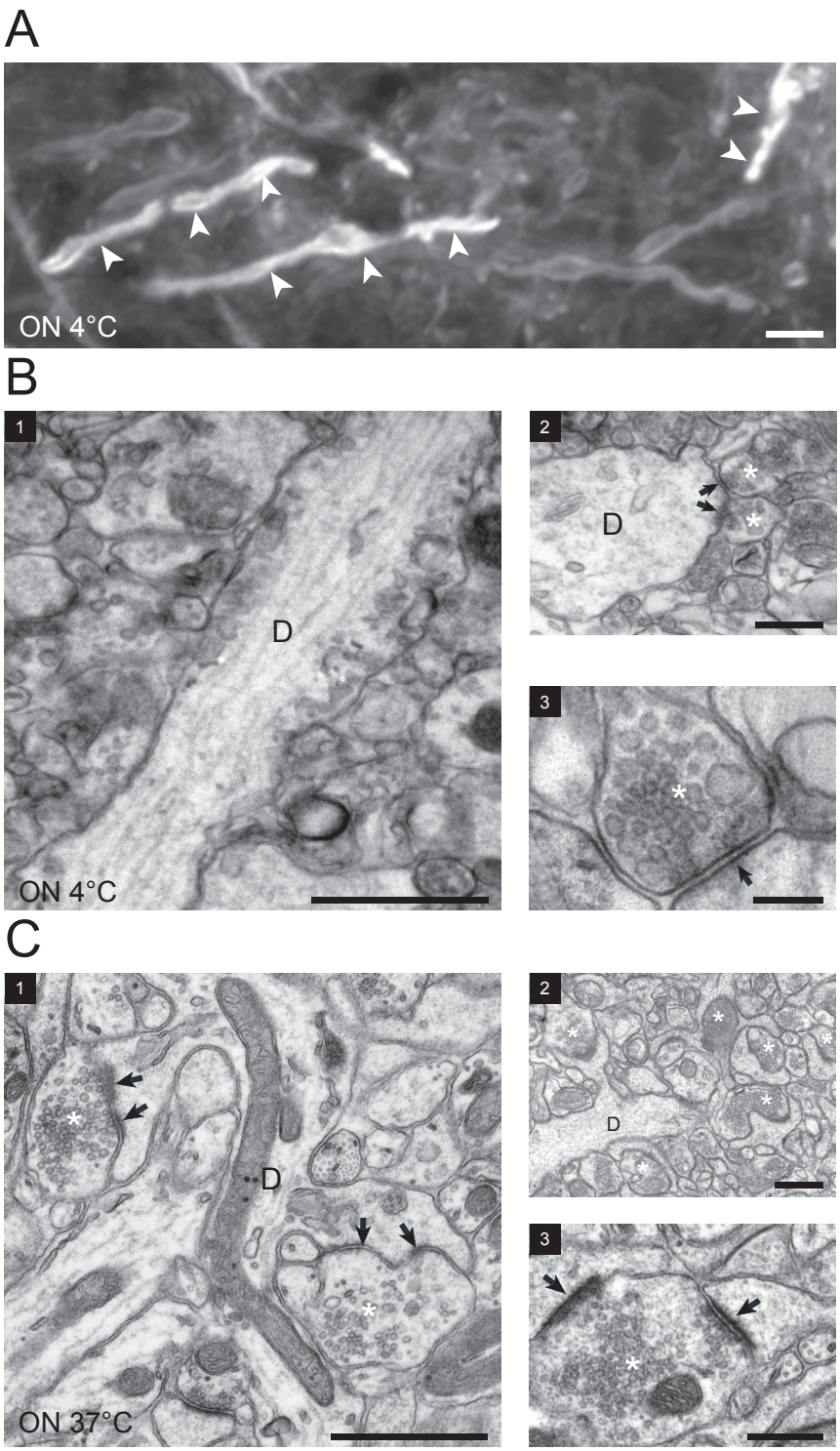


Figure 2: Dendrites in slices cooled to 4°C are devoid of dendritic spines
(a) Acutely cut slices from the brains of adult mice were kept overnight at 4°C, fixed and single neurons were labelled with a lipophilic dye (DiI). Dendrites had lost their spines after the prolonged cooling to 4C° (arrowheads). Bar = 5 µm (b1-3) Ultrastructure of dendrites in acutely cut brain slices from adult mice, incubated overnight at 4°C. Asterisks indicate presynaptic boutons, arrows the postsynaptic density. (b1) Longitudinal cross-section of a dendrite (D) in CA1 that has no spines on its surface. Bar = 1 µm (b2) Synapses on dendrites were made directly onto the shaft of dendrites after cooling. Bar = 0.5 µm (b3) After cooling synaptic junctions often lacked a clear postsynaptic density. Bar = 0.2 µm (c1-3) Ultrastructure of the neuropil in a brain slice of the same preparation as in (b), maintained overnight at 37°C. Asterisks indicate presynaptic boutons, arrows the postsynaptic density (c1) Dendrite bearing two dendritic spines receiving synaptic contacts. Bar = 1 µm (c2) Neuropil ultrastructure is well preserved. Bar = 0.5 µm (c3) Presynaptic bouton synapsing onto two dendritic spines. Bar = 0.5 µm.

using a gene-gun to deliver the membrane marker DiO ('DiOlistic' labeling Gan et al., 2000). Incubating these adult slices overnight at 4˚C resulted in the complete disappearance of dendritic spines (Fig. 2A). Additionally, we performed electron microscopy on the same samples (Fig. 2B1) and found that dendrites in these cooled slices were devoid of spines. Despite the absence of spines, synapses were still present making synaptic contacts directly onto dendrite shafts (Figs. 2B2 and 2B3). In contrast, control slices made using the same technique but maintained overnight at 37˚C showed densely packed neuropil with dendritic spines receiving synaptic contacts at regular intervals along the dendritic shaft (Fig. 2C).

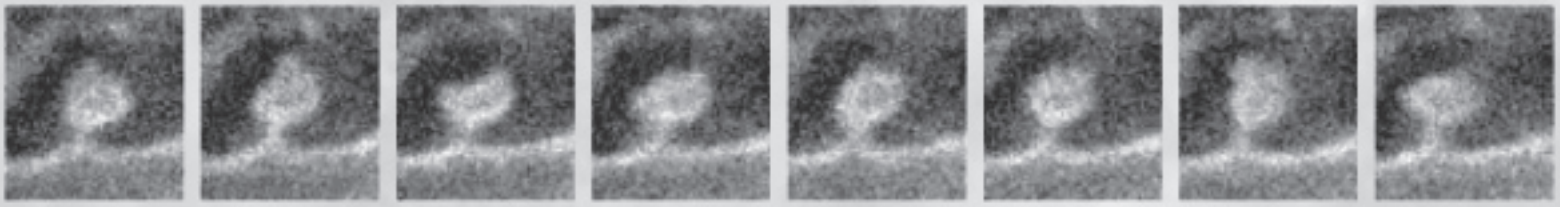
Discussion

In hibernating animals brain temperature can fall to 5˚C and previous studies have indicated that this extreme hypothermia is associated with reversible loss of dendritic spines (Boycott, 1982; Popov et al., 1992). Our data confirm this link between spine structure and reduced temperature, showing a rapid and reversible blockade of morphological plasticity and ultimately loss of dendritic spines when tissue from mouse brain is cooled to room temperature. These observations underline the high degree of plasticity retained by neuronal connections in the mature CNS and suggest that this phenomenon results from temperature-dependent effects on the actin cytoskeleton. Electrophysiological studies on brain slices are commonly carried out at room temperature and our data suggest that spine structure, which influences diverse aspects of synaptic function (Takumi et al., 1999; Euler & Denk, 2001; Helmchen, 2002), is likely to be compromised. Further support for a link between variations in temperature and changes in synaptic morphology comes from a study showing that spillover of extra-synaptic glutamate is reduced at physiological temperature (33 - 36˚C) compared to room temperature (21 - 23˚C) at which such studies are conventionally performed (Asztely et al., 1997). These observations suggest that the extent of spillover depends on temperature-dependent changes in the path length of glutamate diffusion out of the synaptic cleft (Clements, 1996a; Isaacson, 2000), indeed it has been suggested that the deep invagination of the mammalian rod synapse is adaptation to prevent spillover to neighbouring rods (Rao-Mirotznik et al., 1998). Such an arrangement would limit glutamate spillover by extending the diffusion path length out of the synaptic cleft and providing enhanced opportunities for neurotransmitter reuptake via glutamate transporters, an interpretation that is favoured by the observation of Asztely et al. the effects of raising the temperature were partially reversed by the glutamate uptake inhibitor dihydrokainate (Asztely et al., 1997). These factors may relate to our present results

A recent study using tissue from GFPtKras transgenic mice to image the detailed surface structure of dendrites (Roelandse et al., 2003) suggests how this temperature-dependence may relate to our present observations. We found that dendritic spines in living hippocampal slices differ in morphology from the conventionally accepted view of spine structure. Instead of underlying the presynaptic terminal they enclose it to varying degrees (Roelandse et al., 2003), increasing the extent of the synaptic junction in just the manner suggested by models of the balance between glutamate spillover and containment within the synaptic cleft. The regression of spine structure at reduced temperature observed in our present experiments is thus consistent with the finding that spillover is greater at 21 - 23˚C than at 33 - 36˚C (Asztely et al., 1997).

Previous work from our laboratory has shown that dendritic spine motility is blocked by volatile anaesthetics at clinically relevant concentrations (Kaech et al., 1999). Moreover, cooling the brains of animals and humans to 20˚C is known to produce effective anaesthesia independently of chemical anaesthetics (Antognini, 1993; Liu et al., 2001). Together with these previous observations, our present data support suggestions that dendritic spines may play a role in global brain function (Crick, 1982; Matus, 2000).

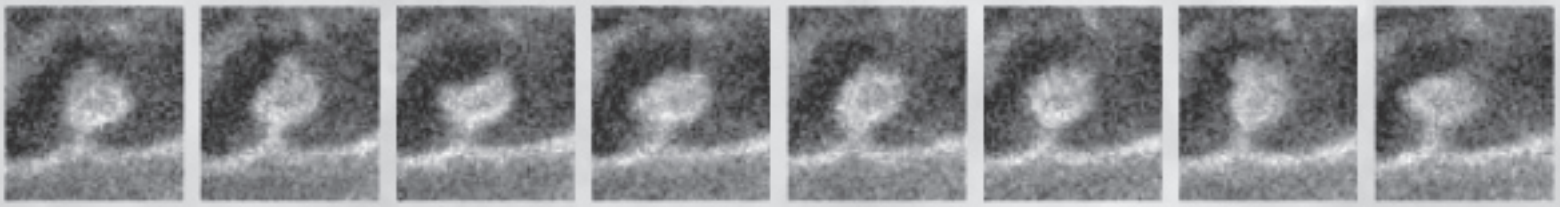
ADDENDUM



I have participated, as a part of my PhD project, in a collaboration between several research groups under supervision of Prof. Matthijs Verhage (CNCR, Amsterdam, the Netherlands). My contribution to the collaboration is summarized underneath and is followed by the joint manuscript that is due to be submitted.

Establishment of connections between neurons is believed to require signals secreted from axons that induce filopodial outgrowth on dendrites and finally synapse formation. We studied the dependence of network formation and maintenance on synaptic activity using munc18-1 null mice. Deletion of this protein leads to a complete loss of neurotransmitter secretion from synaptic vesicles throughout development. Despite this, brain assembly is normal though animals die postnatally. Culturing single hippocampus slices from munc18-1 null mice at embryonic day 18 lead to a complete loss of neurons after 12 days in vitro, suggesting that the absence of synaptic activity is fatal for neurons. To rescue these neurons, we set out to co-culture them with wild-type slices that would, by cross innervating, provide synaptic activity. To evaluate the co-culture system, we first used fluorescent (GFPtKras, a membrane marker) and non-fluorescent (wild-type) hippocampi. These co-cultures are suitable to study both pre- and postsynaptic components of network formation. After 30 days in vitro neurons have formed mature axonal varicosities and dendritic spines that remain present up to 6 months in culture. However, in co-cultures of fluorescent munc18-1 null slices with up to 3 wild type slices, munc18-1 null neurons initially grow neurites into wild type cultures but do not mature. Co-culturing prolonged neuronal lifespan with a week but long-term neuronal survival was not achieved. These results suggest that synaptic activity can partially rescue neuronal survival but the absence of munc18-1 in a still to be discovered pathway or process hinders prolonged development and survival of munc18-1 null neurons.

TROPHIC SUPPORT DELAYS, BUT DOES NOT PREVENT CELL AUTONOMOUS DEGENERATION OF NEURONS DEFICIENT FOR MUNC18-1



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Abstract

Neuronal cells that are deficient for munc18-1, completely lack synaptic vesicle secretion and have severely reduced large dense core vesicle secretion (Verhage et al., 2000; Voets et al., 2001). Munc18-1 mutant neurons develop normally up to the formation of synapses, but eventually degenerate. Here we aimed to isolate the function of munc18-1 that is essential for neuronal viability. Dissociated munc18-1 mutant neurons died within 4 days in vitro (DIV). Application of insulin or BDNF prolonged neuronal viability up to 7 DIV. Culturing on a glial feeder layer allowed mutant neurons to survive up to 10 DIV, or even 14 DIV when insulin was applied. Providing synaptic input by mixing mutant and wild-type neurons in dissociated culture did not enhance the viability of mutant neurons. However, in organotypic culture, afferent innervation did prolong mutant neuron viability from 9 DIV to 19 DIV. Cell specific deletion of munc18-1 in an otherwise normal brain resulted in the specific loss of the munc18-1 deficient cells. Together, these results suggest that munc18-1 mutant neurons do not degenerate due to a lack of neurotransmitter induced activity or trophic support, but because munc18-1 is essential for long-term neuronal viability, regardless of its known role in regulated secretion.

Key Words: apoptosis, loxP, mouse, regulated secretion, trophic support

Introduction

During normal brain development, a surplus of neurons and synapses is formed after which the excess neurons and synapses are eliminated. For synapses there is ample evidence that this elimination process is based on the level of activity and to which extend they contribute to optimal postsynaptic depolarization (Katz & Crowley, 2002; Buffelli et al., 2003). Up until now the precise parameters for neuronal elimination, which occurs cell-autonomously through programmed neuronal death, are unclear, nonetheless, it holds an intuitive appeal that the capacity of a neuron to communicate efficiently with its targets is a main criterion for neuronal survival.

We have previously generated munc18-1 (mammalian uncoordinated 18-1) null mutant mice that completely lack spontaneous and evoked neurotransmitter secretion (Verhage et al., 2000; Heeroma et al., 2003) and have severely compromised large dense core vesicle (LDCV) secretion (Voets et al., 2001). Brain development in munc18-1 null mutant mice is normal up to the point of synapse formation, after which widespread neurodegeneration occurs in developmental order; i.e. early-formed brain regions degenerate first. Degenerating cells in the mutant brain have electron dense nuclei and cytoplasm, condensed chromatin and are TUNEL (terminal dUTP nick end labelling) positive, suggesting that munc18-1 deficient neurons die through apoptotic mechanisms (Verhage et al., 2000).

However, the exact causes for munc18-1 deficient neurons to enter programmed cell death are unknown. One possibility is that mutant neurons trigger their own degeneration because they are critically affected in one of their main functions; the secretion of neurotransmitters, and consequently, the induction of neurotransmitter mediated, postsynaptic electrical activity. Alternatively, munc18-1 mutant neurons could degenerate from a lack of neurotrophic support, for instance because LDCV secretion is impaired. Finally, it is possible that munc18-1 is involved in a cellular process that is indispensable for long-term neuronal survival, but unrelated to its function in regulated secretion.

The aim of this study was to further examine the development of munc18-1 deficient neurons and to isolate the munc18-1 function that is indispensable for neuronal survival. The neocortex and hippocampus are relatively late in brain development and do not show any sign of degeneration at the time of birth. We therefore used neocortical and hippocampal neurons in organotypic, low density and micro island cultures, subjected these neurons to different conditions of activation and trophic support and analysed the effects on munc18-1 deficient neuronal viability. To verify our in vitro data, we also generated a conditional and brain region specific munc18-1 deficient mouse and analysed the fate of munc18-1 deficient neurons in an otherwise normal brain.

Methods

Laboratory animals

Munc18-1 deficient mice were described previously (Verhage et al., 2000). For various experiments, munc18-1 deficient mice were crossed with either mice expressing soluble green fluorescent protein (GFP) (a kind gift

of Dr. A. Nagy) or mice expressing a GFP-based neuronal surface marker (GFPtKRas, Roelandse et al., 2003). Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous mating. Purkinje cell specific knockout mice were created using Cre/loxP-mediated recombination. L7-Cre transgenic mice (Barski et al., 2000) were crossed with loxP-munc18-1 mutants. LoxP-munc18-1 mutants were created by insertion of loxP sites flanking exon 2 of the Munc18-1 gene by homologous recombination in ES-cells. The mouse lines and crosses that were used in each experiment are listed in table 1. Animals were housed and bred in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All animal experiments were approved of by the Dutch Committee for Experiments on Animals and effort was made to minimize animal discomfort.

Cortical cultures

Cortices were dissected from embryonic day 18 (E18) mice and collected in Hanks Buffered Salts Solution (HBSS)(Sigma), buffered with 7 mM Hepes. After removal of the meninges, the cortices were minced and incubated for 20 minutes in trypsinated HBSS at 37° C. After washing, neurons were triturated with fire polished Pasteur pipettes, counted with a hemacytometer and plated in Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% Hepes, 1% glutamax (Invitrogen), 1% Pen/Strep (Invitrogen) and 0.2% β-mercapto-ethanol. Low-density cultures were plated on poly-L-lysine coated glass coverslips at 25,000/cm2. For island cultures, neurons were plated at 6,000/cm2 on islands of rat glia. Glial islands were obtained by spraying a 0.25 mg/ml rat tail collagen solution (BD Biosciences, Bedford, USA) on glass coverslips. After drying and UV sterilization glial cells were plated at 600/cm2. In some experiments, 100 nM insulin (Sigma) was added to prolong the lifespan of munc18-1 deficient neurons. 50% of the medium was refreshed every week.

Organotypic cultures

Organotypic slice cultures from E18 hippocampi were prepared as follows. Mouse embryos were obtained by caesarean section of pregnant females from timed heterozygous mating. GFP-expressing animals were identified by direct inspection using a Leica MZ12 dissection microscope fitted with fluorescence optics. Brains were dissected in ice-cold dissection Gey's balanced salt solution (dGBSS, consisting of GBSS (Invitrogen) with 0.65 g glucose and 200μM kynurenate, pH 7.4) and cut into 400 μm thick slices using a McIlwain tissue chopper (Mickle Engineering, Gomshall UK). After separating individual slices, hippocampal region was dissected out and separated. These hippocampal slices were kept at 4°C in dGBSS for 45 min to recuperate. All subsequent procedures were identical to those described for organotypic slice cultures from P8 mice (Gahwiler, 1998).
For confocal imaging slices were mounted in purpose-built chambers (Life Imaging Services, Olten Switzerland) and observed under continuous perfusion with artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 2.5 mM KCl, 2.0 mM MgSO4, 1.25 mM KH2PO4, 26 mM NaHCO3, 10 mM glucose, 4 mM sucrose and 2.5 mM CaCl2) saturated with 95% O2 /5% CO2 using a Yokogawa microlens Nipkow confocal system (Perkin Elmer, Life Science Resources, Cambridge UK). Images were acquired using a cooled CCD camera (PCO Computer Optics GmbH, Kelheim Germany) and analyzed with MetaMorph software (Universal Imaging Corp., West Chester PA).

Electrophysiological Recordings

Whole cell current-clamp recordings were performed on cultured neurons at DIV 8 and 9. The patch pipette contained the following solution (in mM): 125 K+-gluconic acid, 10 NaCl, 4.6 MgCl2, 4 K2-ATP, 15 Creatine Phosphate, 1 EGTA and 20 U/ml Phospocreatine Kinase (pH 7.30). External medium contained (in mM): 140 NaCl, 2.4 KCl, 4 CaCl2, 4 MgCl2, 10 HEPES, 10 glucose (pH 7.30). Glutamate (1 mM) was added using an application pipette for the duration of 4 seconds (with picospritzer). Axopatch 200A was used for whole-cell recordings. Signal was acquired using Digidata 1322A and Clampex 8.1. Clampfit 8.0 was used for offline analysis.

Immunocytochemical procedures

Dissociated cultures were fixed by adding 4% paraformaldehyde (PFA) to the medium in a 1/1 ratio. After 15 minutes the PFA/medium mixture was exchanged for 4% PFA, for 15 minutes. After washing with PBS the cells were permeated with 0.1% Triton X-100 for 5 minutes. After washing with PBS the cells were ready for processing or storage. Before staining the cells were incubated in 4% foetal calf serum for 20 minutes to block a-specific reactions. After washing with PBS the cells were incubated in a mixture of antibodies and 0.1% Triton X-100 for 1 hour at room temperature or overnight at 4° C. The antibodies used were: mouse anti-MAP2 (1:200, Boehringer Mannheim), mouse anti-HPC1 (syntaxin) (1:1000, Sigma), rabbit anti S100β (1:400, Dako, Glostrup, Denmark). After washing 3 times 5 minutes with PBS, the cells were incubated for 1 hour at room temperature in goat anti-mouse Alexa546 (1:1000, Molecular Probes, Oregon, USA). After washing in 3 times 5 minutes in PBS the coverslips were mounted on microscopic slides with Dabco-Mowiol and analysed with a Zeiss 510 Meta Confocal microscope.
Cultures slices were prepared by microwave-assisted fixation (Jensen & Harris, 1989). Briefly, slices were transferred into pre-warmed fixative (4% PFA, 0.5% glutaraldehyde and 2mM CaCl2 in 0.1M cacodylate buffer (CCB), pH 7.3) and irradiated at maximum power (1000 W) for 9 sec in a Bio-Rad H2500 microwave processor. Post-irradiation temperature, measured by the built-in temperature probe, was 35 - 50° C. Slices were washed 5 x 10 min in 0.1M CCB and incubated overnight at room temperature in 0.3% Triton X-100 5% normal goat serum (NGS; Invitrogen) and 1% bovine serum albumin (BSA; Sigma) in 0.1M CCB. Slices were then incubated overnight at room temperature in 0.1M CCB with 0.3% Triton X-100, 5% NGS, 1% BSA, mouse anti-MAP2C (1:5, Weisshaar, 1992 #335) and rabbit anti-GFP (1:800, Novartis?). After washing 5 times for 10 min each in 0.1M CCB slices were incubated overnight at room temperature in 0.1M CCB with 0.3% Triton X-100, 5% NGS, 1% BSA, goat anti-mouse Alexa546 (1:200, Molecular Probes) and goat anti-rabbit Alexa488 (1:200, Molecular Probes). After washing, slices were stored at 4° C in 0.1M CCB and mounted for imaging in purpose-built chambers, as described above.
L7-Cre × loxP-munc18-1 mutant and wild type mice were anesthetized by Nembutal injection and transcardially perfused with 4% PFA. Histochemistry was performed by incubating cryosections (25 µm thickness) for 3 min in 0.05% Thionine. Sections were analyzed with a light microscope (DM-RB, Leica) equipped with a digital camera. Immunocytochemistry was performed on cryosections (25 µm thickness), rinsed in 0.05 M Tris (Ph 7.6), and preincubated in blocking buffer (5% normal horse serum and 0.25% Triton X-100 in 0.5 M Tris) for 60 min. GAD antibody (sh-a-GAD 1:2000, gift from Oertel) and calbindin antibody (r-a-CaBp

Table 1: Mouse lines and crosses that were used in this study

Crosses	Experimental group	Control group	Experiment
Munc18-1+/- x munc18-1+/-	Munc18-1-/-	Munc18-1+/- munc18-1+/+	Micro island culture, low density (co-culture)
Munc18-1+/-; gfp x munc18-1+/-; gfp	Munc18-1-/-; gfp	Munc18-1+/-; gfp munc18-1+/+; gfp	Micro island culture, low density (co-culture)
Munc18-1+/-; gfptkras x munc18-1+/-; gfptkras	Munc18-1-/-; gfptkras	Munc18-1+/-; gfptkras	Organotypic culture munc18-1+/+; gfptkras
Loxp-munc18-1+/+ x loxp-munc18-1+/-;	Loxp-munc18-1+/+; l7cre+/-	Loxp-munc18-1+/-; l7cre+/-	Conditional cerebellar Purkinje cell specific munc18-1 knockout

1:10000, Swant, Bellinzona, Switserland) were diluted in blocking buffer, and sections were incubated for 24hrs at room temperature. After incubation, samples were rinsed in Tris, followed by 15 min incubation in 0.125% Glutaraldehyde in 0.5M Tris. Incubation with the secondary antibodies Cy3 (d-a-sh, 1:200) and Fitc (d-a-r, 1:200, both from Jackson Immuno Res. Lab. Inc, West grove, USA) was performed in blocking buffer for 2 hr at room temperature. Fluorescence was documented using confocal imaging microscopy (LSM 510 inverted confocal microscope, argon/krypton laser; Zeiss).

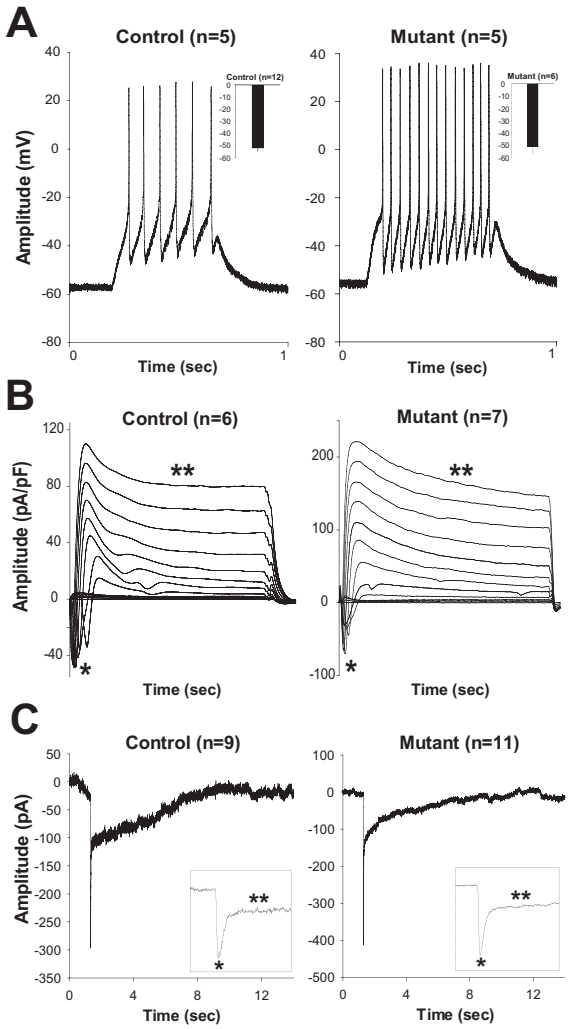


Figure 1: Electrophysiological characterization of munc18-1 deficient neurons in culture
(A) Current injection into cultured neurons induced action potentials in both control and munc18-1 mutant neurons (n = 5). The amount of current injection was not monitored during the recordings. The difference in action potential frequency can thus be explained by the variable amount current injection between recordings. The Vrest of autaptic islands of munc18-1 deficient neurons is very similar to control autaptic neurons (p=0.86; see inset in panel A). (B) The Na+ and K+ channel expression was studied using repetitive block-pulse stimulation (200 ms block pulse; -70 to 40 mV; 10 mV increments). Both control (n=6) and mutant (n=7) neurons show Na+-current (single asterisk, tetrodotoxin-sensitive) and K+-currents (double asterisk, compound current). (C) Example traces of glutamate application inducing inward current in both wild type (n=9) and mutant (n=11) neurons (Vm=-70 mV). Inset: First 6 seconds of amplification. A fast AMPA component (single asterisk) and a slow NMDA component (double asterisk) were observed in recordings from both control and mutant neurons.

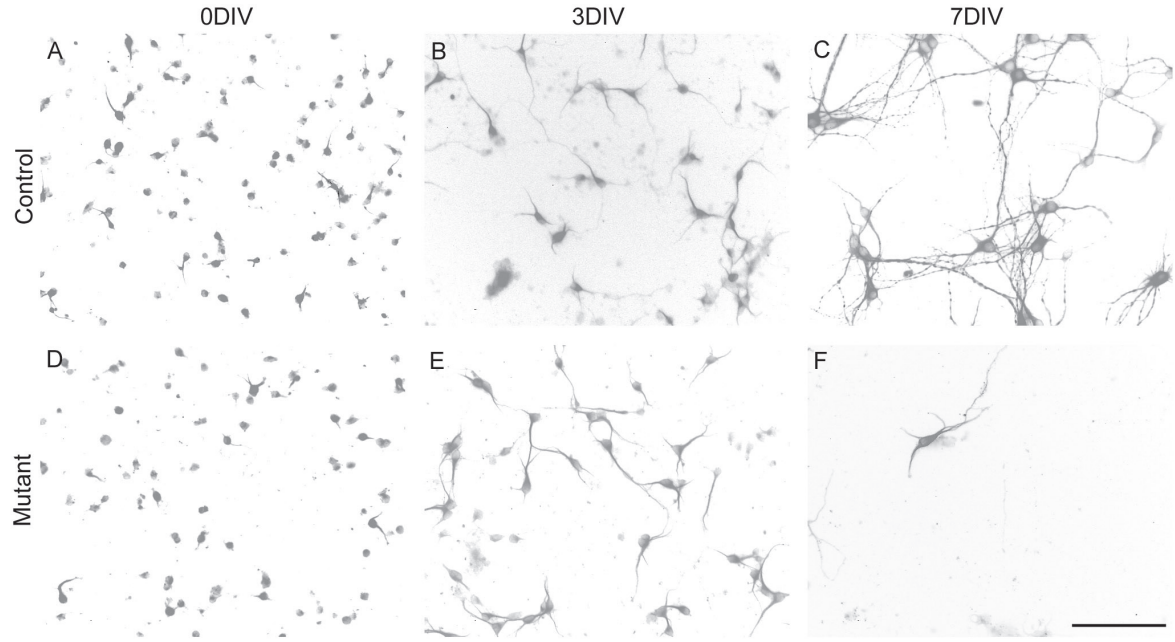


Figure 2: Survival in vitro of munc18-1 null mutant neurons and their controls
Control (A - C) and mutant (D - F) neurons, identified by MAP2 staining, were plated at equal densities. At 3DIV, control (B) and mutant (E) neurons were still in culture at equal densities. At 7DIV, only a fraction of mutant neurons (F) was left in culture compared to the control situation (C). Scale bar is 50 μm.

Results

Munc18-1 deficient neurons lack synaptic vesicle secretion but are otherwise biophysically normal

Munc18-1 deficient neurons are unable to fuse synaptic vesicles (Verhage, 2000 #2099). Here, we investigated whether the absence of munc18-1 affects other biophysical properties of cultured neurons. The resting membrane potential (Vrest) is generally accepted as an informative parameter for the viability/health of (cultured) neurons (Pancrazio et al., 2001). The Vrest of munc18-1 deficient neurons in autaptic island cultures (DIV 8) was normal (Vrest, control= -52.0 mV; Vrest, mutant= -51.1 mV)(Fig. 1A). Current injection into neurons (DIV 8) induced action potentials in both mutant and control neurons (Fig. 1B) and K+(*) and Na+(**) currents were comparable between control and mutant neurons (Fig. 1C).
Previous studies have shown that munc18-1 deficient neurons express functional GABA and acetylcholine receptors (Verhage et al., 2000). Here, we tested whether munc18-1 deficient neurons express functional glutamate receptors. Glutamate application (1 mM, 4 sec) elicited both a fast (AMPA-R mediated) and a slow (NMDA-R mediated) response in control and mutant neurons indicating that both types of glutamate receptors are present and functional in mutant neurons (Fig. 1D). Thus, apart from the lack of synaptic vesicle fusion in munc18-1 deficient neurons, we did not detect any biophysical difference between mutant and control neurons.

Munc18-1 is essential for neuronal viability

Since mutant neocortex and hippocampus show no signs of degeneration at embryonic day (E) 18, these structures were used as substrates for culturing experiments. At E18, both structures were reduced in size in mutant embryos but were otherwise morphologically normal (data not shown). Cortical neurons were plated

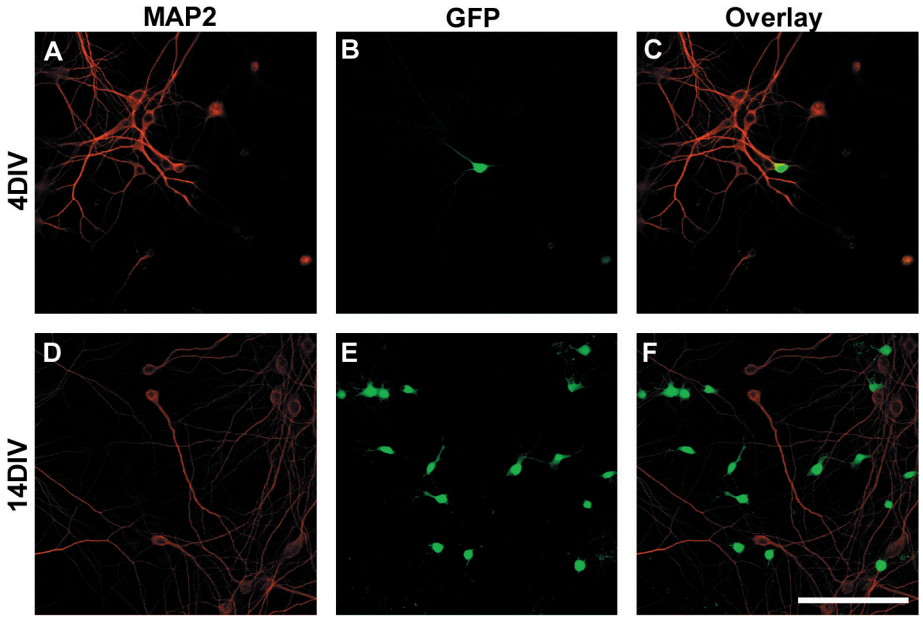


Figure 3: Co-culturing with wildtype neurons does not prolong the lifespan of munc18-1 deficient neurons
Munc18-1 deficient, GFP positive neurons were co-cultured with control/wildtype neurons. After 4div, very few MAP2 positive green cells are left in culture (A-C), suggesting few neurons have survived up to 4div. At 14div, none of the GFP expressing cells is MAP2 positive (D-F), indication that all the munc18-1 deficient neurons have degenerated. Scale bar is 100 µm.

at 20k/cm2. Trypan blue staining indicated that the viability of mutant and control neurons after dissociation was identical (data not shown). As another measure for neuronal viability after dissociation, neuronal attachment to the substrate was analysed at 2, 6 and 24 hours after plating. At all three time-points, more than 95% of both mutant and control neurons were attached (data not shown), suggesting no difference in initial viability in culture between both groups.

After 3 DIV, the amount of control and mutant neurons was counted in 24 randomly chosen fields of 0.137 mm2. For control neurons the average count (+/- standard deviation) was 21.5 +/- 6.7. The count for mutant neurons was on average 22.2 +/- 5.4 per field. This corresponds to densities of 15.7k/cm2 and 16.2k/cm2 for control and mutant neurons respectively. This suggests that approximately 20% of both control and mutant neurons are lost between 1 and 3 DIV.

After 7 DIV, neurons were again counted in 48 randomly chosen fields of 0.137 mm2. Control culture average

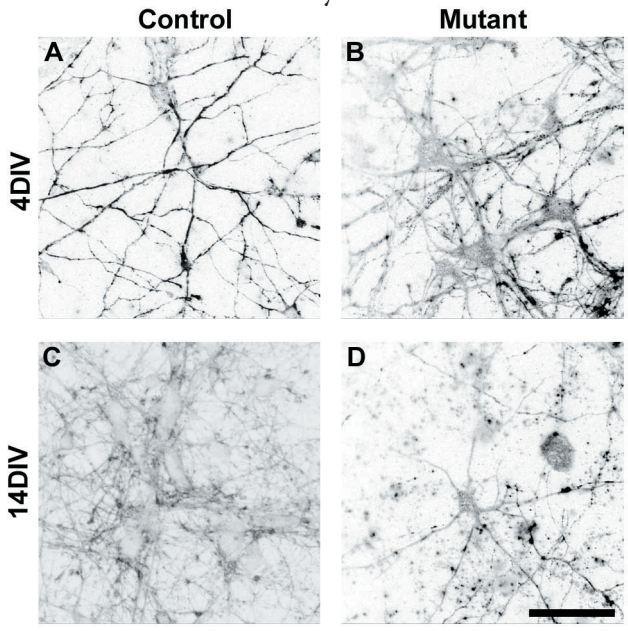


Figure 4: Addition of insulin delays the degeneration of munc18-1 deficient neurons.
100 nM insulin was added at div0, and cultures were stained for syntaxin to control for neuronal presence. After 4div, munc18-1 deficient neurons (B) were present in normal numbers and with similar morphology as control neurons (A). After 7div, only a fraction of the munc18-1 deficient neurons had survived (D). The morphoogy of these cells was comparable to control neurons (C). The scale bar is 50 µm.

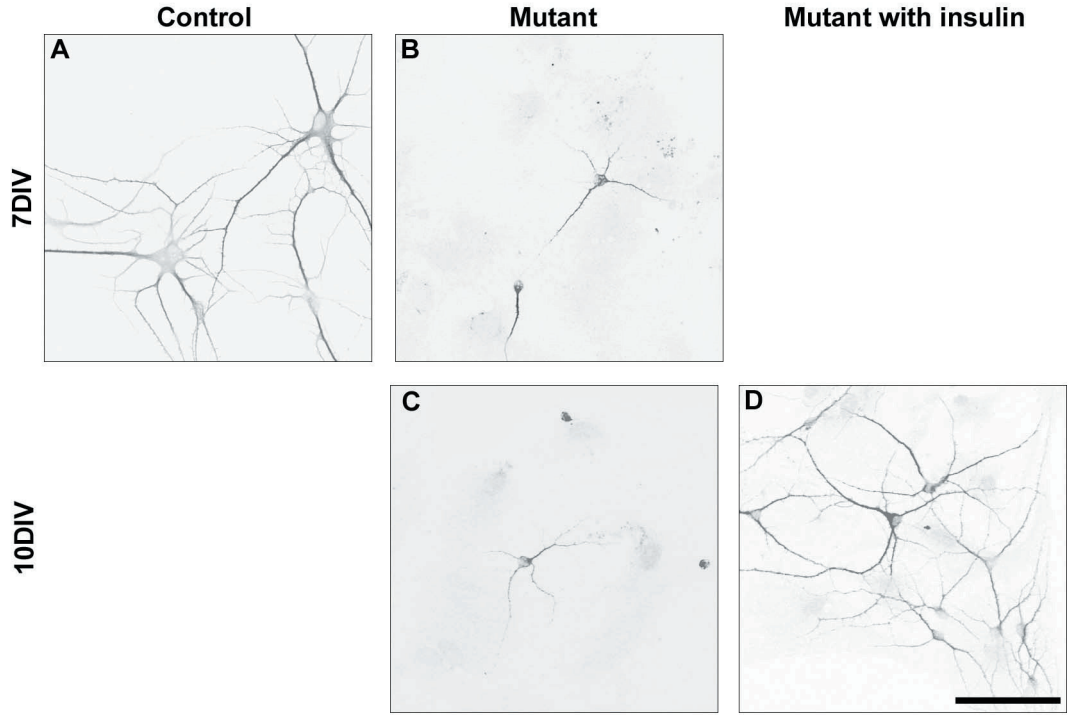


Figure 5: Culturing on glial cells further delays the degeneration of munc18-1 deficient neurons.
Neurons were plated on glial feeder layers and stained for MAP2 to identify neuronal presence. After 7div, munc18-1 deficient neurons (B) were still present in numbers comparable to control neurons (A). However, mutant neurons have shorter neurites and less branching points. After 10div, some mutant neurons (C) are still present, but most have degenerated. When insulin is added to these cultures (D), normal morphology is preserved and mutant neuronal viability is stretched to ~12div. The scale bar is 50 □m.

density was 15.4 +/- 4.9 neurons whereas in mutant cultures the average was 0.7 +/- 0.7 neurons per field. Thus, while control neurons at 7 DIV were still present at a density of 11.2k/cm2, the majority of mutant neurons had died by 7 DIV. This corresponds with findings from routine visual inspection where the typical 7 DIV survival rates of control neurons are approximately 50 % against 0.01 to 0.001 % for mutant neurons.
This indicates that munc18-1 deficient neurons, obtained from E18 neocortex, are equally viable as control neurons during the first 3 DIV. However the massive loss of munc18-1 deficient neurons between 3 and 7 DIV suggests a critical role for munc18-1 in neuronal viability in later stages.

Co-culturing munc18-1 deficient neurons with wild type neurons does not improve neuronal viability

The degeneration of munc18-1 deficient neurons may be due to a lack of synaptic input from other neurons and could thus be mitigated or even prevented by mixing mutant and wild type neurons in low-density culture. To identify the different neuronal populations, the munc18-1 null mutation was crossed into transgenic mice expressing green fluorescent protein (GFP) under control of the cytomegalovirus (CMV) promoter (see table 1). Co-cultures were prepared using munc18-1 deficient GFP expressing neurons and non-fluorescent control neurons and, vice versa, of GFP expressing control neurons together with non-fluorescent munc18-1 deficient neurons. At 3 DIV, the development of munc18-1 deficient neurons expressing GFP was indistinguishable from those without GFP, regardless of the presence of control neurons. At 4 DIV, few mutant neurons, identified by microtubule associated protein-2 (MAP2) staining, remained in co-culture (Fig. 3A-C). At 5 DIV, approximately 0.001 % of mutant neurons remained in co-culture, similar to mutant neurons in mono-culture. At 14 DIV, some GFP-positive cells were still in culture, but these cells lacked MAP2 staining (Fig. 3D-F), and were probably glial cells (discussed below). Thus, culturing munc18-1 deficient neurons with wild type neurons has

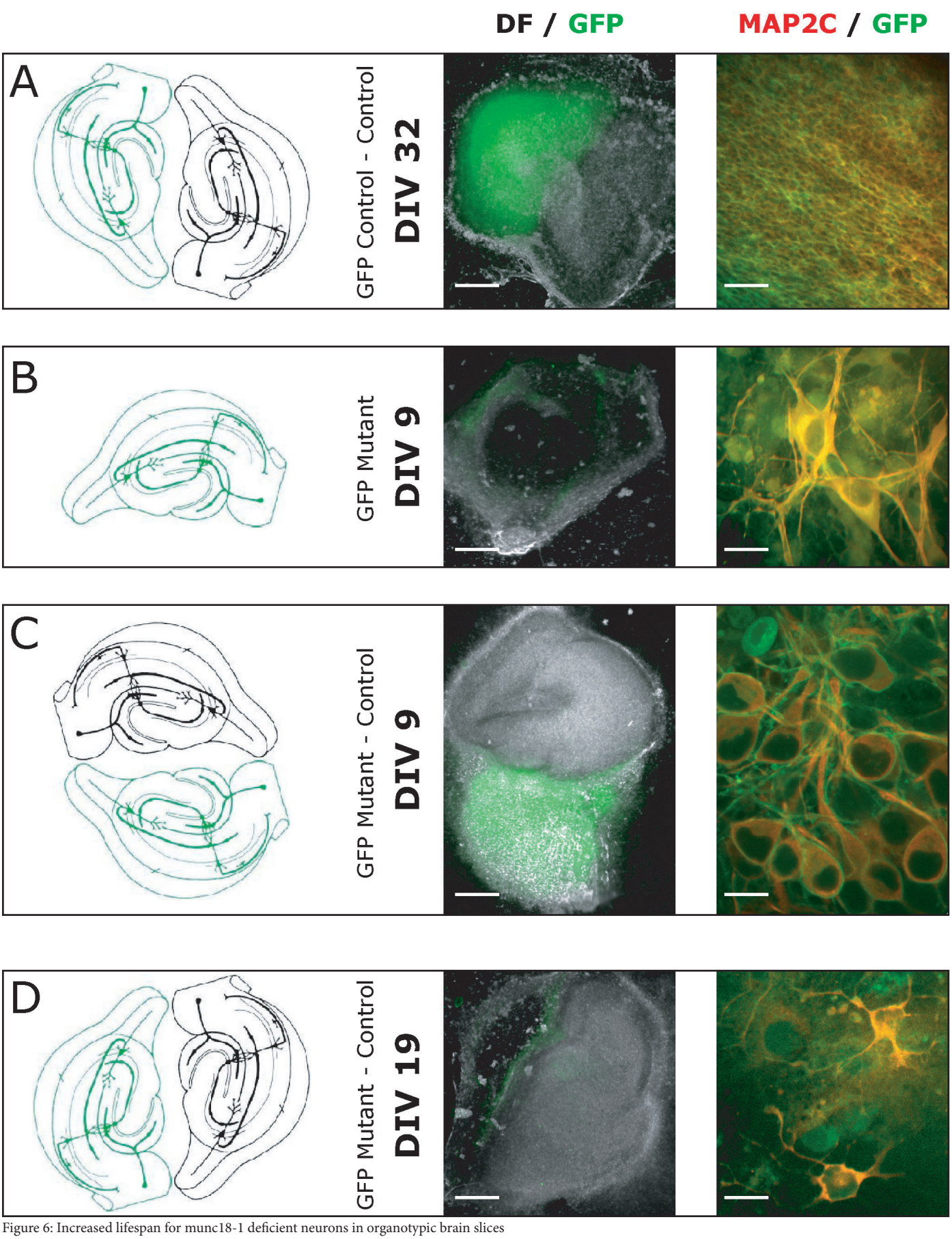


Figure 6: Increased lifespan for munc18-1 deficient neurons in organotypic brain slices
(A) At 32DIV the gross morphology of co-cultures of fluorescent and non-fluorescent wild type mice is normal (left panel). In wild type slices expressing GFPtKras at 9DIV neurons in area CA1 are densely packed (right panel). (B) Single munc18-1 deficient slice culture at 9DIV (left panel) in which some MAP2 positive neurons are present (right panel). (C) Co-culturing munc18-1 deficient slices with non-transgenic wild type slices for the same length of time prevents neuronal degradation (left panel). Many MAP2 positive neurons can be found in the munc18-1 deficient slice (right panel). (D) Ultimately the munc18-1 deficient slice deteriorates (left panel) with only a few surviving munc18-1 deficient neurons left (right panel). Bar figs. A-D, left panels = 300 ⊠m / bar figs. A-C, right panels = 40 µm / fig D, right panel = 100 µm.

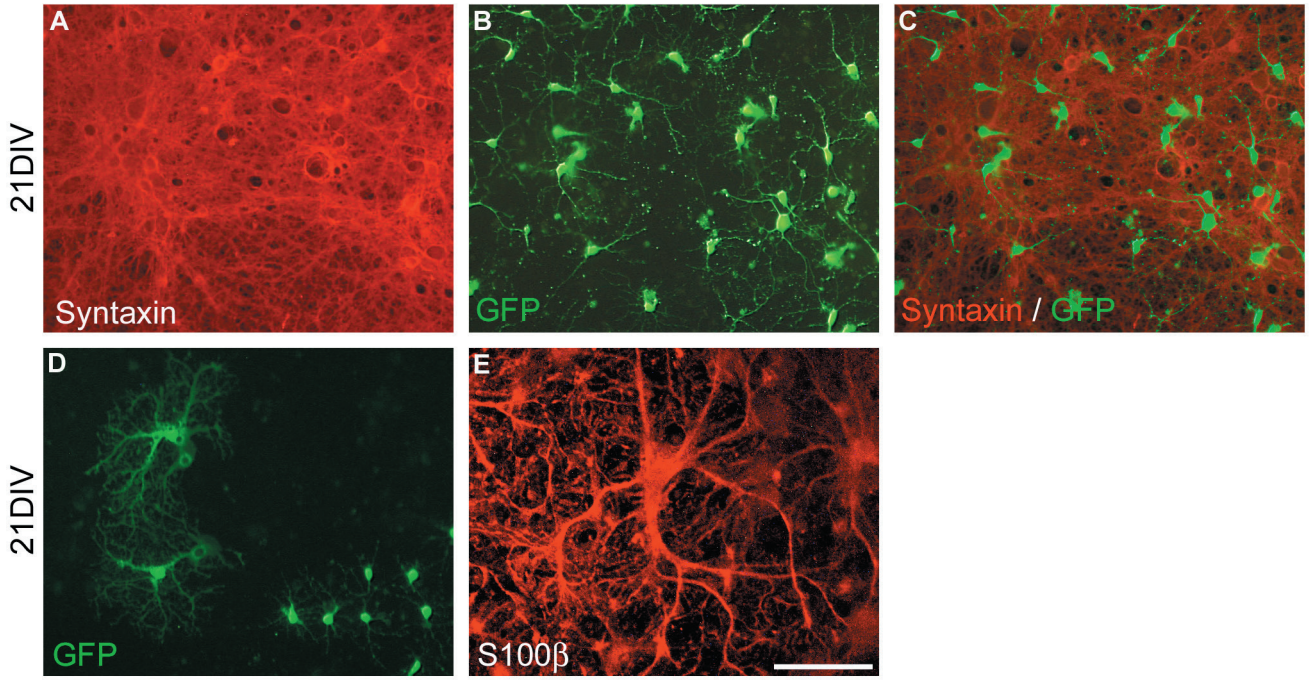


Figure 7: Munc18-1 deficient glial cells remain in culture
. When control and munc18-1 deficient – GFP expressing cortical cells are co-cultured for 21div, no munc18-1 deficient neurons are left in culture. There is extensive syntaxin staining, showing the presence of a dense neuronal network (A). There are a number of GFP expressing cells indicating that some munc18-1 deficient cells are still present (B). However, the GFP expressing cells show no structural overlap with the syntaxin staining (C) and therefore are not syntaxin positive/of neuronal origin. When munc18-1 deficient cortical cells are plated in mono-culture, two cell types survive (D). Some of these remaining cells are S100 positive and resemble type 2 astrocytes (E). The other cells have the morphological appearance of oligodendrocytes. The scale bar is 50 m.

no noticeable effect on neuronal viability.

Trophic factors delay the degeneration of munc18-1 deficient neurons

Deletion of munc18-1 expression does not only completely block neurotransmitter secretion, but also has a profound negative influence on the secretion of LDCVs (Voets et al., 2001). Since LDCVs often contain trophic substances that promote neuronal viability or modulate neuronal function, addition of trophic and/or neuromodulatory substances to the culturing medium might alleviate the phenotype of munc18-1 deficient neurons. Brain derived neurotrophic factor (BDNF) and insulin (-related proteins) are known to have a trophic and survival promoting effect on neurons (Lindholm et al., 1996; Yamada et al., 2001). Addition of 100 nM of insulin to the culturing medium prevented the massive loss of munc18-1 deficient neurons at 4 DIV (Fig. 4A, B); no difference was observed in neuronal density of munc18-1 deficient and control cultures. Insulin supported munc18-1 deficient neuronal cultures up to 7 DIV (Fig. 4C, D). The number of munc18-1 deficient neurons that reached 7 DIV, however, was lower (approximately 1%) than in control cultures (approximately 50%) and between 7 and 11 DIV, the remaining neurons degenerated. Addition of BDNF to the culturing medium had a similar effect on mutant neuron viability (data not shown). Thus, insulin and BDNF delay, but do not prevent, degeneration of munc18-1 deficient neurons.

Culturing on glial cells further delays the degeneration of munc18-1 deficient neurons

Not only neurons can give trophic support; most neuronal support in the brain is provided by glial cells (Barres, 1991). Therefore, in mutant neurons, glial support might compensate for the loss of munc18-1. To test this hypothesis, control and munc18-1 deficient neurons were cultured on a glial feeder layer. After 7 DIV, the

number of munc18-1 deficient neurons in culture (Fig. 5B) was comparable with the control situation (Fig. 5A) indicating that glial support indeed had a positive effect on the viability of munc18-1 deficient neurons. However, the appearance of these neurons was clearly different from control neurons; dendrites were smaller and the number of branching points was reduced in munc18-1 deficient neurons (for details see Heeroma et al., in preparation). After 10 DIV on a glial feeder layer, approximately 1% of munc18-1 deficient neurons were still in culture (Fig. 5C). Similar to the situation at 7 DIV, these neurons appeared morphologically underdeveloped and none survived past 10 DIV. Interestingly, when insulin was added to munc18-1 mutant neurons cultured on glia, neuronal morphology and survival rate were still normal at 10 DIV (Fig. 5D). However, after 10 DIV degeneration still occurred and no mutant neuron reached 14 DIV. Together, these data suggest that glial cells and insulin have a synergistic effect in promoting the viability of munc18-1 deficient neurons. In addition, insulin aids munc18-1 deficient neurons to obtain/maintain normal neuronal morphology. Nevertheless, glial cells and insulin, neither separate nor in combination, prevent degeneration of mutant neurons.

Munc18-1 deficient neuronal viability increases in organotypic brain slices

As trophic substances and glial cells worked synergistically in delaying degeneration of munc18-1 deficient neurons embedding of these neurons in a more natural trophic environment might delay this degeneration even further. To test this, we used organotypic slice cultures of E18 hippocampus. The munc18-1 null allele was crossed into mice transgenically expressing GFPtKras that has been shown previously to be a useful tool to study morphological analysis (Roelandse et al., 2003).

Previous publications have shown that postnatal brain slices can be kept in vitro for more then 4 weeks and that neuronal development in these slices closely resembles in vivo maturation (Gahwiler, 1984). Since munc18-1 deficient mice are postnatally lethal, embryonic day 18 mice were used for generating the slice cultures. Co-cul-

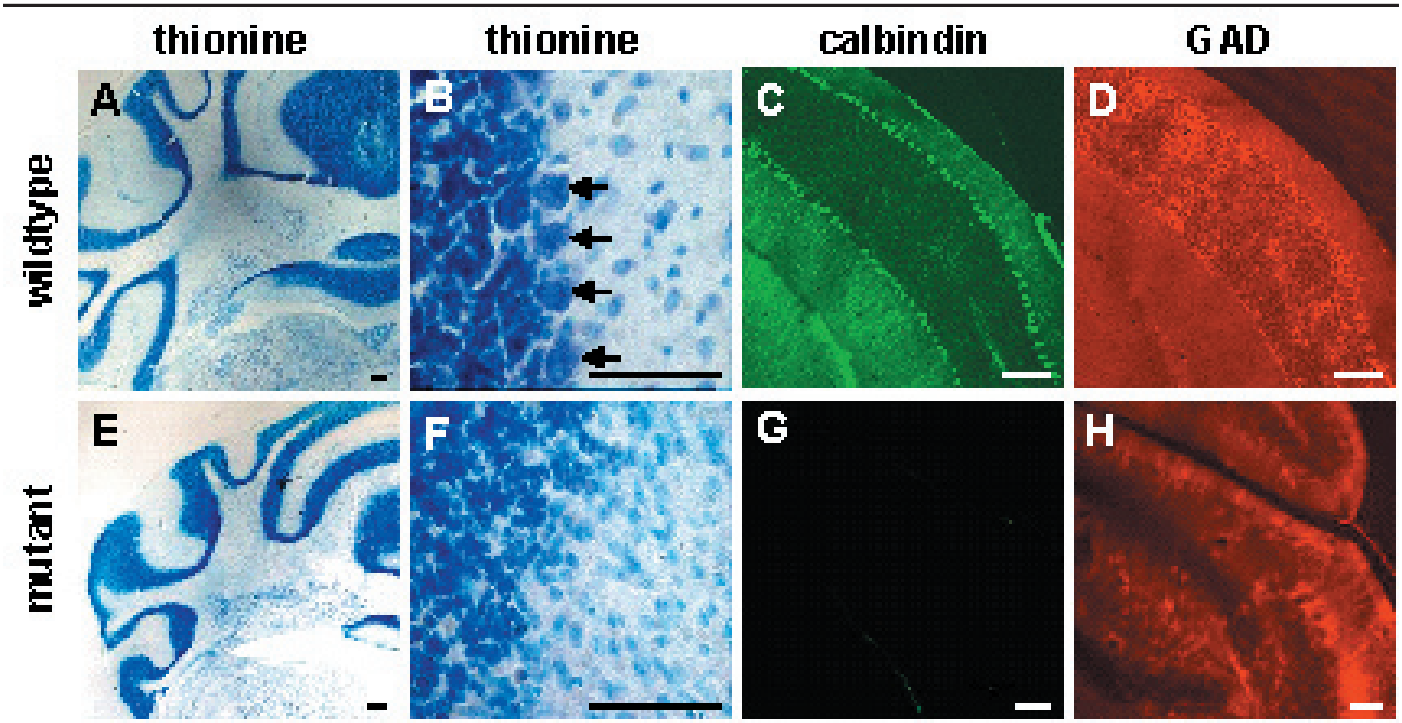


Figure 8: Neurons lacking munc18-1 in vivo do not remain viable.
The cre-lox system was used to specifically delete munc18-1 in post-natal cerebellar purkinje cells (E-H). Nissl staining showed relatively normal cerebellar anatomy in adult mutant mice (E) compared to wildtypes (A). Purkinje cells (arrow) were present in wildtypes (B) but absent in mutants (F). Calbindin is a positive marker for Purkinje cells (C). Mutants showed no calbindin positive cells (G). GABA-ergic interneurons and purkinje cells are positive for GAD (D). Mutant interneurons were positive for GAD (H). The scale bar is 100µm.

tures of fluorescent and non-fluorescent wild type slices are known to cross-innervate and to develop mature neuronal characteristics as dendritic spines and presynaptic varicosities (Roelandse and Matus, in preparation). Such a culture at 32 DIV is shown in figure 6A (left panel); both hippocampi were equal in size and retained morphological features characteristic for mature hippocampus.

Maintaining single munc18-1 deficient slices expressing GFPtKras in culture resulted in a rapid degradation of tissue during the first week (Fig. 6B, left panel). At 9 DIV, some neurons could still be identified using MAP2C-staining (Fig. 6B, right panel). However, at later stages, all neurons had degenerated, suggesting that organotypic cultures per se are equally successful in preventing the degeneration of munc18-1 deficient neurons than combined glial and trophic support in island cultures.

Mixed low-density cultures of munc18-1 deficient and control neurons did not delay the degeneration of mutant neurons. Interestingly, co-culturing munc18-1 deficient slices, expressing GFPtKras, with non-fluorescent control slices did delay the degradation of the fluorescently labelled munc18-1 deficient slice (compare Figs. 6B and C, left panels). At 9 DIV, munc18-1 deficient neurons were present in the co-culture in numerous amounts (Fig. 6C, right panel) whereas they were virtually absent at that stage in single munc18-1 deficient slices. At 19 DIV, the GFP expressing munc18-1 deficient slice had degenerated, despite the presence of a control slice (Fig. 6D, left panel). Still, some MAP2 positive neurons were present in the vicinity of the non-fluorescent wild type culture (Fig. 6D, right panel) nonetheless no neurons could be observed beyond 20 DIV. Together, this suggests that not organotypic slice culturing per se, but co-culturing with control slices prolongs the lifespan of the munc18-1 deficient neurons.

Munc18-1 deficient glial cells remain in culture

Although different extracellular environments increase the lifespan of munc18-1 deficient neurons to various extends, eventually every neuron that lacked munc18-1, died prematurely (i.e., before control neurons died). Still, some munc18-1 deficient, GFP positive and MAP2 negative cells survived to 14 DIV in mixed cultures (Fig. 3D-F), suggesting these cells were not neurons. Figure 7B shows that these cells were still present after 21 DIV, were mitotic (data not shown) and syntaxin-1 negative (Fig. 7A, C). By visual inspection, two cell types could be distinguished among the remaining cells (Fig. 7D). One cell type closely resembled the morphology of type 2 astrocytes and this was supported by the finding that a subset of these cells was S100β positive (Fig. 7E). The other cell type morphologically resembled oligodendrocytes. These data indicate that, in contrast to neurons, glial cells are not affected by the absence of munc18-1.

Timed and cell specific deletion of munc18-1 in vivo causes specific loss of munc18-1 deficient neurons

Since munc18-1 deficient embryos die upon birth, cultured neurons were used to analyse the effects of munc18-1 deletion on neuronal viability. Hence, it is possible that the in vitro environment obscures the actual in vivo phenotype of munc18-1 deficient neurons. In addition, the apoptotic environment of the mutant embryonic neurons might cause misinterpretation of the actual mutant phenotype. To minimize these risks, a mouse was generated in which munc18-1 could be conditionally excised using the cre-lox system. This munc18-1-lox

mouse was crossed with a mouse, transgenically expressing cre under control of the L7 promoter, which is specifically active in cerebellar Purkinje cells. Cre mediated excision of munc18-1 starts at postnatal day 6 and is complete in all Purkinje cells at postnatal week 3 (Barski et al., 2000). To allow depletion of remnant munc18-1 protein, the effect of munc18-1 deletion was analysed after 8 postnatal weeks. Contrary to their littermates, animals homozygous for the munc18-1-lox allele, had developed severe ataxia by this time, indicating some cerebellar defect (data not shown). Nissl staining showed that overall cerebellar morphology of mutant mice (Fig. 8E) was indistinguishable from control mice (Fig. 8A), but closer examination showed that Purkinje cell bodies were absent in the mutant cerebellum (Fig. 8B, F). Staining for Purkinje cell marker calbindin showed immunoreactivity in layer 4 of the cerebellum of control mice (Fig. 8C) but was completely absent in the cerebellum of mutant mice (Fig. 8G). Finally, staining for glutamic acid decarboxylase (GAD), a general marker for inhibitory neurons, showed increased immunoreactivity in the Purkinje cell layer of control cerebelli (Fig. 8D), but not of mutant cerebelli (Fig. 8H). Together, these results suggest that cell specific deletion of munc18-1 results in the specific loss of those neurons that lack munc18-1.

Discussion

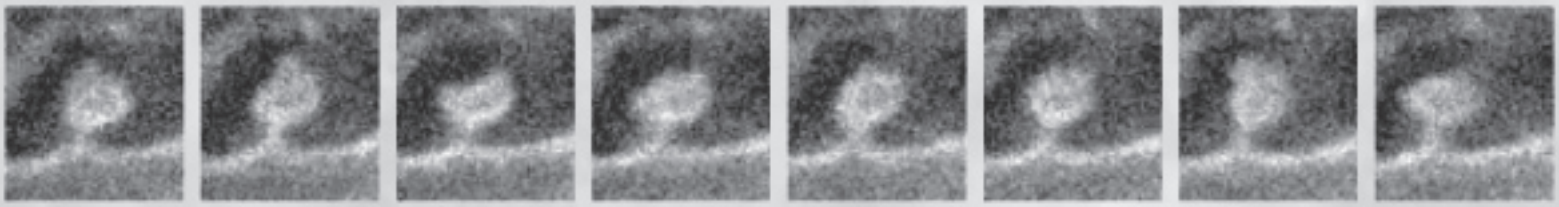
In the present study, we have shown that munc18-1 deficient neurons, which are incapable of synaptic vesicle fusion, have otherwise normal biophysical properties, but degenerate in vivo and in micro island, low density and organotypic cultures. However, providing trophic substances, glial support and afferent innervation, can delay, but not prevent, this degeneration in a synergistic manner.
As described before (Verhage et al., 2000), degeneration of munc18-1 deficient neurons starts in the embryonic brain and follows developmental order. The finding that control and mutant neocortical neurons are equally viable during the first 3 DIV strongly suggests that mutant neurons were healthy at the time of plating and had not yet started to degenerate. The fact that mutant neurons can remain in culture for up to 20 DIV supports this.

One of the primary neuronal functions is communication through the secretion and subsequent reception of neurotransmitters. Since the total lack of vesicular neurotransmitter secretion is the most conspicuous effect of munc18-1 deletion, this may be causally related to the degeneration of mutant neurons. However, providing afferent input by co-culturing mutant and control neurons in low density did not prevent or delay mutant neuron degeneration. Curiously, providing afferent input in mutant organotypic cultures delays degeneration up to 10 DIV, suggesting a positive effect on neuronal viability. The main difference between organotypic and low density cultures is the dissociation procedure, which inevitably sets back the neuronal developmental program, and consequently may cause afferent input to be less effective in delaying degeneration of mutant neurons in low density culture. In a recent report, neurons deficient for both munc13-1 and munc13-2 (mammalian uncoordinated 13-1 and 2 respectively) were maintained in micro island culture for 12 DIV without any neuronal degeneration (Varoqueaux et al., 2002). Similar to munc18-1 deficient neurons, munc13-1/2 double knockout neurons are incapable of synaptic vesicle fusion and thus lack functional afferent and efferent contacts. It is therefore unlikely that neurotransmitter secretion or reception is critical to neuronal survival, although the delayed degeneration of munc18-1 deficient neuron in organotypic co-cultures does suggest a beneficiary effect of afferent input on neuronal viability.

In chromaffin cells, deletion of munc18-1 decreases LDCV secretion by approximately 90% (Voets et al., 2001). By analogy, LDCV secretion could be affected in central neurons as well. Treatment of munc18-1 deficient neurons with insulin or BDNF delayed degeneration by approximately 3 DIV. This might imply that munc18-1 deficient neurons degenerate due to defective secretion of neurotrophic substances. Contrary to the munc18-1 mutant, LDCV secretion is not reduced in munc13-1/2 deficient chromaffin cells (personal communication Brose/Rosenmund?). Therefore, the difference in viability of neurons lacking munc18-1 and munc13-1/2 may be caused by their differential capacity to fuse LDCVs.
Although delaying neuronal degeneration, application of insulin or BDNF did not rescue the deletion of munc18-1. Possibly, a better spatial and temporal acuity, dosage and mixture of neurotrophic substances, that is normally directed by regulated secretion, is essential for neuronal survival.

The trophic actions of insulin and BDNF are multiple; both substances reportedly stimulate neurite outgrowth, synapse formation, synapse activation and synaptic strength (Schulingkamp et al., 2000; Vicario-Abejon et al., 2002). However, both substances are also known to exert survival-promoting effects (Yamada et al., 2001; Hamabe et al., 2003). Therefore, the delay in munc18-1 deficient neuronal degeneration might be an effect of temporary suppression of apoptosis. Still, this does not explain the prolonged survival of mutant neurons on a glial feeder layer, or the synergistic effect of glial cells and insulin.
Cell specific deletion of munc18-1, using the cre-lox system in vivo, resulted in the specific loss of cerebellar Purkinje cells. Altogether, this suggests that munc18-1 deficient neurons do not degenerate due to a lack of afferent innervation but rather are prone to degenerate, regardless of their direct environment or capacity for regulated secretion. Therefore, we propose that munc18-1 has other, unknown, functions that are not related to regulated secretion, but are essential for long-term neuronal survival.

GENERAL DISCUSSION



The previous four chapters highlighted some of the fascinating aspects of neuronal hardware, ranging from the morphological development of postsynaptic specializations and their fundamental behaviour, to presynaptic differentiation of axonal varicosities and the subsequent complexity of age-dependent synapse consolidation. I have also tried to illustrate the plasticity of mature neuronal networks and further assessed the role of activity using some bugs such as hypothermia and mice transgenically engineered to be incapable of neurotransmitter release. Some of the questions outlined in the aims of this study may have successfully been resolved however for others the answer is still incomplete or absent due to unexpected setbacks.

Development and plasticity of neuronal networks

Past neuroscience research has shed much light on the various aspects of both developing and mature brain in order to ultimately understand its complex structure and behaviour thereby being able in due course to ameliorate malfunctioning and mental disorders (Albright et al., 2000). Still, many aspects of basic development and neural network formation remain unclear.

Dissociated cell cultures have proven their use as a tool for studying the process of neuronal navigation as a result of their easy accessibility in combination with the well-defined cellular environment in addition to the minimal requirement of bright field imaging setup. Fundamental questions, such as how a sprouting neurite becomes an axon or how growth cones eventually reach their target and what defines their turning behaviour, could herewith partially be answered (for review, see Song & Poo, 2001; da Silva & Dotti, 2002; Dickson, 2002). It is however clear that the reality of neuronal pathfinding in vivo is far more complex; here growth cones are likely to be controlled by the balanced effects of the various repulsive and attractive guidance cues. The introduction of green fluorescent protein and its spectral variants that were originally derived from the jellyfish Aequorea victoria, to illuminate molecules of ones interest (for review, see Matus, 2001) in combination with the cultures mentioned above have successfully been used to further understand the process of synaptogenesis (Jontes & Smith, 2000; Cohen-Cory, 2002; Garner et al., 2002). Nonetheless, it remains unknown what signal (-ling molecule) is responsible for determining the location of a newly formed synapse and despite suggestive evidence using retrograde staining of functional synaptic elements (Friedman et al., 2000) and the subsequent discovery that both axons and dendrites extend filopodia to look for potential pre- or postsynaptic partners (Dailey & Smith, 1996; Wong & Wong, 2000; Chang & De Camilli, 2001; Roelandse et al., 2003), neither is the temporal order of arrival of pre- and postsynaptic elements.

Finally, only a few years back it was discovered that dendritic spines undergo rapid and spontaneous shape changes using the actin-based cytoskeleton (Fischer et al., 1998). Later data suggested a certain degree of plasticity for axonal varicosities (Hatada et al., 2000; Colicos et al., 2001; Luthi et al., 2001; De Paola et al., 2003). Together it is likely that these dynamics in pre- and postsynapse influence signal transmission between neurons.

In contrast to most past research described above, I have exploited organotypic cultures, in which in vivo neuronal development is closely mimicked, of previously generated transgenic mice expressing either actin-GFP (Fischer et al., 2000) or GFP bearing a surface membrane localization signal (Roelandse et al., 2003) in combination with confocal live cell imaging system, together providing me a useful tool for studying the

relationship between actin dynamics and shape changes of both pre- and postsynaptic structures during development. Furthermore, GFP attached to the plasma membrane in combination with real-time fluorescence imaging without bleaching or phototoxicity, resulted in a high signal-to-noise ratio and hence high resolution images of axon and dendrite fine structure that enabled us to capture subtle shape changes in contrast to other markers used for live cell imaging, such as soluble GFP (e.g. Dunaevsky et al., 1999; Engert & Bonhoeffer, 1999; Maletic-Savatic et al., 1999) or lipophilic dyes (e.g. Bolz et al., 1990; Molnar & Blakemore, 1991; Shepherd et al., 2002).

The presented data on E18-E18 co-cultures show a subsequent focalisation of actin-based dynamics in both axons and dendrites but at the same time suggest a high degree of preservation of morphological plasticity in mature neuronal networks. Both pre- and postsynaptic compartments in the mature mammalian CNS would thereby retain a capacity for experience-dependent fine-tuning e.g. during either periods of learning and memory or during brain damage resulting in an altered connectivity similar to those observed in vivo (Kaas, 1991; Gilbert, 1993; Buonomano & Merzenich, 1998; Yuste & Sur, 1999). In addition, the fate of axons growing into a wild-type slice seemed to depend on the age of the tissue when the cultures were established. Whereas co-cultures of E18 hippocampus exhibit sustained cross-innervation forming a dense plexus with the majority of axons still present after 1 month in vitro, co-cultures of P8 hippocampus were very different. Despite the intensive formation of axonal projections during the first two weeks in culture, a dramatic regression of axons was observed over time resulting in a near complete absence after 1 month in vitro. A possible explanation for this phenomenon may lie in the time of culturing. At P8, most axonal afferents have established functional connections however due to the culturing procedure, dentate gyrus granule cells will inevitably loose their afferent innervation which is then subsequently replaced by CA1 afferents (Gutierrez & Heinemann, 1999). It may therefore be that once axon fibres reach the co-culture after several days in vitro, most neurons by now receive sufficient afferent stimulation and hence synaptic connections. Assuming pyramidal cells in both slices to be equipotent, very few postsynapses will then change presynaptic partner since the existing connection is not likely to be worse and may even be strengthened. If it were true that synaptic activity is essential for postsynaptic fine-tuning, maintaining P8-P8 co-cultures in the presence of drugs that inhibit all neurotransmitter release, such as Botulinum toxin, may then prevent the formation of functional or strengthened synapses and may therefore result in a dense cross-innervation as observed in E18-E18 co-cultures since both ingrowing and resident axons have equal opportunities to form strong connections once the BoTX is removed. P8-P8 co-cultures could also be treated with AMPA receptor antagonists since recruitment of these receptors to the membrane may coincide with the late period of synaptic fine-tuning (Liao et al., 2001) and therefore may result in a subsequent strengthening of connections.

To investigate whether the observed difference in capacity to cross-innervate were the result of tissue maturation, heterochronic co-cultures from E18 and P8 slices were setup but irrespective of which slice was labelled, the P8 slice degenerated so that after 2 months in culture it had almost disappeared whereas the E18 slice matures without noticeable problems. Staining these cultures with a microglia marker gave a possible explanation for this unexpected phenomenon; microglial cells had massively invaded the dying P8 slice. However, it remains unclear whether the microglia are the cause of neuronal degradation or are merely "just" a side effect. If microglia were indeed responsible for neuronal degradation in P8 tissue, blocking their proliferation using mitosis-inhibitors such as Ara-C (Svensson & Aldskogius, 1993; Gould & Goshgarian, 1999), or blocking their activation using immuno-modulators such as transforming growth factor (TGF)-β1 (Eyupoglu et al., 2003a;

Milner & Campbell, 2003) would then prevent the P8 slice from degrading and neurons might then develop normally. If neuronal degeneration still occurs in the presence of any of the above-described drugs the occurrence of microglia would suggest being a side effect, likely there to clear up dead cells and debris.

A possible mechanism for cell death in the P8 slice of an E18-P8 culture is the involvement of the $p75^{NTR}$ neurotrophin receptor (Dechant & Barde, 2002), activated either via presynaptically secreted NFG (Haubensak et al., 1998; Heerssen & Segal, 2002) or via microglia surface bound NGF (Frade & Barde, 1998). If the $p75^{NTR}$ receptor is involved, then co-culturing heterochronic slices from wild-type and $p75^{NTR}$ knockout mice (Naumann et al., 2002) would result in the healthy maturation of both the P8 and E18 slice. Culturing P8 and E18 slices in separate plasma clots sharing the same medium would proved a means to maintain heterochronic tissue without any physical contact between cells of different ages. This would both prevent E18 microglia to invade P8 tissue and at the same time exclude miscommunication between cells due to differential expressing of signalling molecules expressed on either postnatal or embryonic cells. If however the P8 slice still deteriorates during neuronal maturation in shared medium, this would suggest the involvement of as yet unknown proteins secreted by E18 cells, which induce the subsequent degeneration of P8 neurons. Seeding (activated) embryonic microglia on top of P8 cultures could be used to test the hypothesis that these cells play a causal role in neuronal degeneration by microglia, a proposition that would be supported if P8 neurons subsequently degenerate. Finally, as speculated earlier, it is also possible that a difference in neuronal activity between the two slices results in the subsequent death of P8 neurons. If this were the case then treating heterochronic cultures with BoTX might result in normal neuronal maturation in both cultures.

The main advantage of using co-cultures of hippocampal slices from fluorescent and non-fluorescent embryonic hippocampal slices is the ability to examine simultaneously dendrite differentiation in the fluorescent slice and to track the fate of fluorescent axons growing into the non-fluorescent slice. Our intention was to follow the chronology of synapse formation and stabilization with the possibility to later intervene in the establishment of neuronal circuits as illustrated using mice that lack neurotransmitter release (Verhage et al., 2000). However, instead of using munc18-1 null mice, that have deficiencies beyond to the lack of neurotransmission limiting their survival, other animals exist that are incapable of releasing neurotransmitter into the synaptic cleft, owing to the lack of an essential protein involved in vesicles release machinery (munc13 - Varoqueaux et al., 2002; SNAP-25 - Washbourne et al., 2002b), yet do not have the observed neuronal degeneration that occurs in munc18-1 null mice. Inbreeding their locus into GFPtKras mice would enable us to further explore the process of synapse formation and maintenance using the previously described organotypic co- or even triple-cultures (as described by Molnar & Blakemore, 1991) in such a way that fluorescently labelled releasing and non-releasing axons invade either side of a non-fluorescent slice. If synaptic activity were essential for synapse formation, ingrowing non-releasing axons would be out-competed by ingrowing wild-type axons in the middle non-fluorescent tissue, which would be easy to track due to the fluorescent marker on the ingrowing axons. An interesting aspect to the scenario described above would be whether transgenic neurons, whose axons are out-competed by wild type afferent innervation capable of releasing neurotransmitter and hence incapable of forming presynaptic connections, would die despite the afferent wild-type innervation they receive themselves. This would suggest that the neurons require for cell survival not only regular and sufficient stimulation of their postsynaptic receptors but also an unknown signal originating from the axon, confirming to have sufficient synaptic connections as has been suggested for postsynaptic connections (Raisman & Field, 1990). On the other hand, if synaptic activity is essential for synapse maintenance, afferents incapable of releasing neuro-

transmitter would initially invade the non-fluorescent culture and form axonal varicosities yet these synaptic connections are then subsequently taken over by axons capable of releasing neurotransmitter. This would leave us with a scenario similar to that observed for P8-P8 co-cultures, where axonal projections initially form but are gradually lost over time. To confirm that the ultimate elimination of non-releasing axons is due to synapse competition, as observed in the neuromuscular junction (Walsh & Lichtman, 2003), labelling the postsynaptic side with one of the spectral variations of GFP would facilitate. Live cell imaging of such cultures would enable a detailed study of synaptic maintenance thereby possibly illuminating axonal competition for dendritic connections that can be further ameliorated using various receptor agonists and antagonists or generalized neurotransmitter blockers.

Finally, having found the successive focalisation of neurite dynamics during development and the long-term stability of synaptic connections in mature tissue, the preserved capacity for morphological plasticity in mature neuronal networks was clearly illustrated in the hypothermia simulations. When mature hippocampal slices, either acutely cut brain slices or organotypic cultures, were cooled to room temperature, dendritic spines first became immotile then disappeared within 12 hours. Nonetheless, rewarming these cooled slices to 37˚C resulted in the rapid extension of filopodia from the surface of dendrites and re-establishment of dendritic spines within several of hours. With regard to others (e.g. Lendvai et al., 2000; Grutzendler et al., 2002) and ours results, it is appealing to propose dendritic spines to have a more global role in brain function and there is some evidence support this hypothesis. First, the correlation between altered dendrite morphology and mental retardation (Marin-Padilla, 1975; Kaufmann & Moser, 2000; Ramakers, 2000) suggests the normal nature of dendritic spines to be of vital importance for cognitive function. Second, changing normal body temperatures either way resulted in the absence of consciousness in healthy patients (Jimenez-Mejias et al., 1990; Lumlertgul et al., 1992; Liu et al., 2001) as well as in the lack of spine dynamics, both during cooling and heating (personal communication I. Brünig). Furthermore, addition of volatile anaesthetics to pyramidal neurons at clinically relevant concentrations for general anaesthetics resulted in an arrest of dendritic spine motility (Kaech et al., 1999) as well as to the lack of consciousness in healthy patients (Schneider et al., 2003). The correlation between the absence of spine motility and state of consciousness suggests that the observed morphological plasticity of excitatory synapses in the brain may be essential for short-term brain function. In summary, these observations suggest that dendritic spines may have a global role in brain function.

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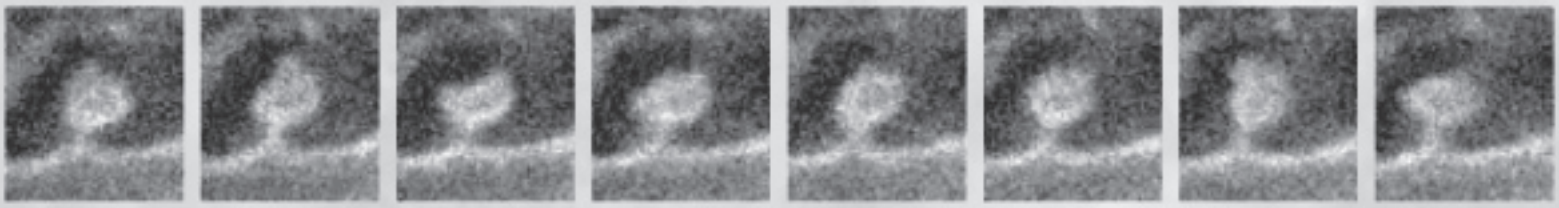

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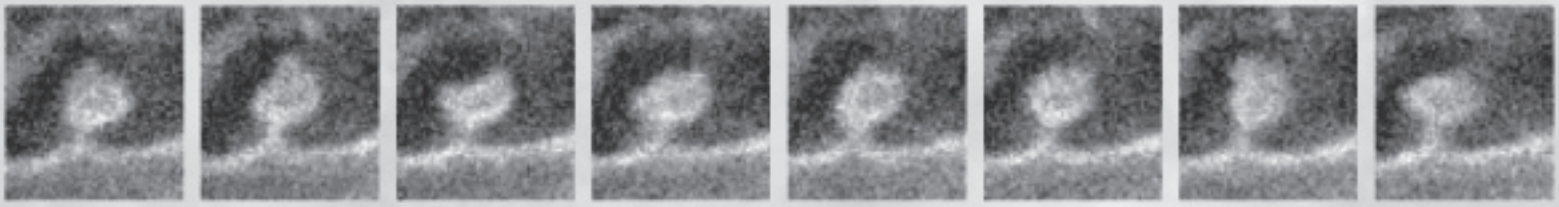

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Martijn

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Dutch NeuroFederation

Publications

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