

Dendritic Cytoskeletal Protein Expression in Mental Retardation: An Immunohistochemical Study of the Neocortex in Rett Syndrome

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Many syndromes associated with mental retardation (MR) are characterized by cortical dendritic anomalies. Despite their morphological similarity, these changes appear to involve different stages of dendritic development. The neuronal cytoskeleton, which includes microfilaments, neurofilaments and microtubules, is essential for these developmental processes. Levels and phosphorylation of microtubule-associated proteins (MAPs), which stabilize microtubules, seem to determine different stages of dendritic formation with certain MAPs (e.g. MAP-2) appearing to mediate the effects of external modulators upon these processes. Early studies on neuronal cytoskeleton in MR, which have shown a selective reduction in MAP-2 expression, have focused on Rett syndrome (RS). Here, by a semiquantitative immunohistochemical analysis of the pericentral cortex, we examine the contribution of specific neuronal populations to these changes in cytoskeletal proteins. Decreased MAP-2 staining in RS was more marked in layers V–VI, while increased nonphosphorylated neurofilament immunoreactivity was found in layers II–III in RS. Age-related increases in dendritic MAP-2 immunoreactivity in layers V–VI were also absent in RS. The specificity of these cytoskeletal protein changes, their significance for RS pathogenesis and plasticity, as well as their implications for other MR-associated disorders, are also discussed.

Introduction

One of the most consistent abnormalities involving the cerebral cortex of individuals with mental retardation (MR) is a reduction in dendritic arborizations (Huttenlocher, 1991; Kaufmann, 1996; Kaufmann and Moser, 2000). In addition, dendritic spines may also be abnormal in morphology and density (Marin-Padilla, 1972; Purpura, 1974; Hinton *et al.*, 1991). Although these dendritic changes are morphologically similar, cross-sectional studies of Down syndrome (DS) and Rett syndrome (RS) suggest that these abnormalities occur at different stages of dendritic development in each condition. For instance, in RS dendritic anomalies are detected as early as clinical diagnosis is made in this disorder and no marked changes take place thereafter (Armstrong *et al.*, 1995). In contrast, in DS dendritic branching and length appear to be normal or increased in infants less than 6 months old but become progressively reduced after 2 years (Takashima *et al.*, 1981; Becker *et al.*, 1986). Dendritic spine abnormalities in unclassified (Purpura, 1974) and genetic MR, such as DS (Marin-Padilla, 1972) and fragile-X syndrome (FraX) (Hinton *et al.*, 1991; Kaufmann and Reiss, 1999), also suggest an arrest at distinct stages of spine formation. Considering this, a strategy for gaining insight into dendritic pathology in MR should examine dendritic components that not only play a critical role in the function of this neuronal process, but also have characteristic developmental profiles. Cytoskeletal proteins fulfill these requirements. They are abundant, with distinctive ontogeny, and are relatively easy to detect by current immuno-

chemical techniques. Furthermore, recent advances in neurobiology emphasize that dendrites are not passive postsynaptic receptive areas, but play an important role in neuronal signal processing (Barinaga, 1995), and that the neuronal cytoskeleton is essential in these dendritic functions (Kaech *et al.*, 1996).

Dendrites and Neuronal Cytoskeleton

The neuronal cytoskeleton consists of three main components, which in order of size are microfilaments, intermediate filaments and microtubules (Kunze and Rüstow, 1993). Each one of these structures is formed by a major protein: actin, neurofilament and tubulin, respectively. Microfilaments and microtubules also have a wide array of associated proteins that modify their physical properties and molecular associations. Although all major cytoskeletal components are present throughout the neuron, cytoskeletal proteins located in somas and dendrites differ, to some extent, from those found in axons (Mitchison and Kirschner, 1988). Therefore, studies of neuronal cytoskeletal proteins usually assess both somatic and dendritic components. Each main cytoskeletal protein would serve specific functions. Neurofilaments (NFs) that consist of three different proteins, termed according to their molecular weight (mol. wt) as low (NF-L, ~70 kDa), medium (NF-M, ~150 kDa) and high (NF-H, ~200 kDa), are the most stable components which provide mechanical support to neurons (Kunze and Rüstow, 1993). In addition, NF concentration and molecular state would influence both neurite (axonal and, perhaps, dendritic) caliber and perikaryal size (Hoffman *et al.*, 1988). The latter is an important feature considering that, in developmental disorders, decreases in dendritic arborizations are frequently associated with smaller neuronal perikarya (Kaufmann *et al.*, 1998; Kaufmann and Moser, 2000). Despite their stability, NF expression and conformation can be modified by neuronal stimulation. For instance, NF-H levels and degree of phosphorylation, which correlate with axonal stabilization and pruning, are increased by neurite membrane stimulation (Riederer, 1992). NFs in their nonphosphorylated form are predominantly localized to cell bodies and dendrites, while phosphorylated NFs are typically found in axons (Shaw and Weber, 1982; Kaufmann *et al.*, 1997a).

The actin microfilament network is a dynamic component of the cytoskeleton, which is greatly influenced by external and intraneuronal stimuli. The balance between actin monomers (G-actin) and polymers (F-actin) is a complex process that mediates changes in cell shape (Kunze and Rüstow, 1993). There are proteins that modify this critical polymerization/depolymerization balance. Salient among these is the actin depolymerizing factor (ADF)/cofilin family (Bamburg, 1999). These proteins bind F-actin cooperatively, inducing a twist in the actin filament that may be responsible for an increase in the off rate of actin subunits (depolymerization) (Bamburg, 1999). These processes

may be essential during development, since ADF/cofilins concentrate in the dynamic actin pools of neuronal growth cones (Bamburg, 1999). As many cytoskeletal and cytoskeletal-associated proteins, ADF/cofilins are modulated through phosphorylation by specific kinases. One of these enzymes is LIMK1 (Stanyon and Bernard, 1999), whose gene is contained in the characteristically microdeleteted region of chromosome 7 in Williams syndrome (WS) (Monaco, 1996; Tassabehji *et al.*, 1999). WS is due to submicroscopic deletions on chromosome 7q11.23, a region that also comprises the elastin and the *HPC-1/syntaxin 1A (STX1A)* genes [reviewed by Monaco, Bellugi *et al.* and Botta *et al.* (Monaco, 1996; Bellugi *et al.*, 1999a; Botta *et al.*, 1999)]. Patients with WS exhibit, in addition to MR, a distinctive cognitive and social phenotype with relative preservation of language and face processing abilities, impairment in visuospatial domains, and hypersociability (Bellugi *et al.*, 1999a,b). LIMK1-dependent phosphorylation of cofilin 'turns off' the actin-depolymerization action (Stanyon and Bernard, 1999) of this protein (Bamburg, 1999). This process is also regulated by Ras-like protein pathways. For instance, Rac-GTP enhances phosphorylation of both LIMK1 and cofilin, leading to an accumulation of F-actin (Stanyon and Bernard, 1999). The precise role of LIMK1 mutation in the WS phenotype is still unknown (Tassabehji *et al.*, 1999). Another link between G proteins and cytoskeleton (including actin) organization is provided by the PAKs (p21-activated kinases) and ROKs (Rho-binding kinases). These proteins seem to mediate the effects of Rho-GTPases on cell growth and differentiation (Leung *et al.*, 1998). An example of their potential role in MR is the subgroup of nonsyndromic X-linked MR that is associated with point mutations in the *PAK3* gene. *PAK3* encodes a serine/threonine kinase that links Rho GTPases to cytoskeletal organization and nuclear signaling (Allen *et al.*, 1998). The precise consequences of *PAK3* are still unknown; however, it is highly expressed in developing and mature cortical neurons. Further emphasizing the potential role of Ras-like proteins in developmental disorders is our report on Rheb, a Ras-like protein that is expressed in dendrites and is activated as an early gene during cortical development and long-term potentiation (LTP) (Kaufmann and Worley, 1999).

The actin network also plays a critical role in neuronal signaling through its close association with the plasma membrane via actin-binding proteins (e.g. α -actinin), and other proteins located in this dense network (Clark and Brugge, 1995), usually termed 'cell cortex'. Among these 'cortical' proteins are dystrophin, the protein product defective in Duchenne muscular dystrophy, and spectrin (Kunze and Rüstow, 1993). Since approximately 30% of the patients with this neuromuscular disorder have MR (Moser, 1995), disorders of the neuronal cellular cortex may underlie developmental disabilities. In support of this hypothesis is the demonstration that dystrophin is expressed in neuronal somas and axon hillocks, in a pattern that resembles spectrin (Torelli *et al.*, 1992). Spectrin appears to play a critical role in synaptic plasticity, as suggested by its change in expression in models such as long-term potentiation (LTP) (Seubert *et al.*, 1988). In addition to neurotransmitter receptor-mediated plasticity, actin seems to play a role in signaling complexes linked to adhesive interactions (between integrins and extracellular matrix). Actin as well as other cytoskeletal proteins may also participate in the process of anchoring protein kinases (e.g. protein kinase C, or PKC), key enzymes in neuronal signaling, facilitating their intracellular localization and activation (Mochly-Rosen and Gordon, 1998).

Microtubule-associated Proteins: Key Cytoskeletal Elements in Dendritic Development

The third major cytoskeletal complex, the microtubular system, is probably the most interesting in terms of dendritic development and function. Microtubules are relatively more abundant in neurons than in other cell types, which may serve as a base for the complex structure of neurons [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. Microtubules are cylindrical polymers, formed by heterodimers of α and β tubulin, which undergo rapid changes in length and stability. As with actin, the dynamic properties of microtubules are modulated by associated proteins termed microtubule-associated proteins, or MAPs (Black and Kurdyla, 1983; Tucker, 1990) [reviewed by Johnson and Jope (Johnson and Jope, 1992)]. Microtubules are essential for axonal and dendritic stability; these processes retract when neurons are exposed to drugs that induce microtubule depolymerization, a phenomenon which is greatly regulated by the levels and conformation of MAPs (Matus *et al.*, 1986) [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. MAPs are divided into two main groups: structural MAPs, which include severing proteins and catastrophe factors, and the more abundant microtubule assembly-promoting proteins. The term MAP is usually referred to the latter class, which modulates polymerization, bundling and stability of microtubules (Black, 1997; Sanchez *et al.*, 2000). The second group of MAPs are motor proteins that are involved in transport of vesicles or proteins along microtubules (dynein or MAP-1C, kinesins) (Tucker, 1990) [reviewed by Johnson and Jope and Sanchez *et al.* (Johnson and Jope, 1992; Sanchez *et al.*, 2000)]. Structural MAPs may also have a role in cell signaling; for instance, MAP-5 (also known as MAP-1b) links certain subtypes of GABA receptors to the cytoskeleton at retinal synapses (Hanley *et al.*, 1999). MAPs are differentially distributed in neuronal processes; for instance, the high mol. wt (or 'adult') form of MAP-2 is almost exclusively in perikarya and dendrites while Tau is predominantly compartmentalized to axons (Matus *et al.*, 1986; Tucker, 1990; Johnson and Jope, 1992; Kanai and Hirokawa, 1995). Other MAPs, such as MAP-1 (also termed MAP-1a) and MAP-5, are present in similar proportions in all neuronal compartments (Tucker, 1990) [reviewed by Johnson and Jope (Johnson and Jope, 1992)].

Dendritic development is composed of two basic phases: an exploratory phase, in which a motile growth cone explores the substratum, and a consolidation phase of growth, in which more stable processes which grow in a preferred direction are formed (Mitchison and Kirschner, 1988). Each one of these phases is characterized by distinct profiles of cytoskeletal proteins. Motile growth cones contain mainly unpolymerized tubulin and, therefore, few microtubules that are essentially unstable (Kaech *et al.*, 1996). During the consolidation phase, growth cones become neurites with thick and stable bundles of microtubules containing acetylated and detyrosinated (polymerized) tubulin associated with MAPs (Black *et al.*, 1986; Mitchison and Kirschner, 1988; Kaech *et al.*, 1996). These distinct stages of microtubule organization are greatly influenced by the interaction between MAPs and tubulin. For instance, recent data indicate that early MAP-2 expression is critical for stabilizing directional dendritic growth, while at the same time maintaining microtubule dynamics which allows dendritic branching (Kaech *et al.*, 1996). Although specific roles in neurite development are understood only for a few proteins, such as MAP-5 and MAP-2 (Ma *et al.*, 1999; Ramon-Cueto and Avila, 1999) [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)], the ontogenetic profile of many cytoskeletal proteins is already known. Postmitotic

neuroblasts express neuron-specific class III tubulin (Menezes and Luskin, 1994), whereas the subsequent phase of neurite growth is correlated with the expression of MAP-5 and the 'juvenile' or low mol. wt form of MAP-2 or MAP-2c (Tucker, 1990; Johnson and Jope, 1992). MAP-5, as many other cytoskeletal constituents, is a phosphoprotein, and has a higher level of phosphorylation during the most active phase of dendritic growth (Ramon-Cueto and Avila, 1999). MAP-2c, an alternatively spliced form of the adult or 'mature' MAP-2, is found in neuronal somas and neurites from the beginning of dendritic extension to the phase of dendritic maturation, as well as, during adult life, in regions of active synaptic remodeling (e.g. olfactory bulb) [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. NF-L and MAP-1 are present at the beginning of dendritic growth or expansion (Shaw and Weber, 1982; Tucker, 1990), while high mol. wt MAP-2 characterizes dendritic expansion and maturation (Matus *et al.*, 1986; Kaufmann *et al.*, 1997a) [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. Finally, NF-H in its nonphosphorylated form appears in the late stages of dendritic formation, when stabilization and elimination of neurites takes place (Shaw and Weber, 1982; Kaufmann *et al.*, 1997a). In addition to the stage-specific onset of expression, cytoskeletal protein levels follow characteristic ontogenetic curves. For instance, MAP-5 and MAP-2c levels decrease substantially towards the beginning of dendritic expansion and maturation with relatively stable levels thereafter (Johnson and Jope, 1992). In contrast, NF expression increases progressively up to a plateau that coincides with dendritic pruning (Kaufmann *et al.*, 1997a). Both levels and degree of phosphorylation seem to be the most important factors regulating the function of all MAPs, particularly their role in microtubular organization (Sanchez *et al.*, 2000). In correspondence, posttranslational changes in MAPs and other cytoskeletal proteins are also characteristic of the process of dendritic formation. MAP-5 is phosphorylated at early ages (Matus *et al.*, 1986; Riederer, 1992; Ramon-Cueto and Avila, 1999) while MAP-2 phosphorylation of an amino-terminal epitope seems to increase with age, coinciding with distal dendritic extension (Riederer, 1992; Riederer *et al.*, 1995). On the other hand, the overall level of MAP-2 phosphorylation seems to decrease throughout neuronal maturation [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. Although the precise role of MAP-2 phosphorylation in microtubular organization is still under investigation, this posttranslational change has been linked to both early microtubule formation and late stabilization. The latter effects seem to depend more on the specific residues that are phosphorylated than on the overall level of phosphorylation. Nevertheless, phosphorylation of different domains of MAP-2 leads to reduced tubulin binding by either conformational changes and/or by direct modifications of the tubulin-binding domain [reviewed by Johnson and Jope and Sanchez *et al.* (Johnson and Jope, 1992; Sanchez *et al.*, 2000)]. Based on these findings, Sanchez and colleagues (Sanchez *et al.*, 2000) have postulated a general model for the role of MAP-2 in dendritic development. During the initial phase of dendritic outgrowth, an imbalance in favor of kinases over phosphatases would lead to higher levels of phosphorylated MAP-2, predominantly MAP-2c. The decreased microtubular binding of phosphorylated MAP-2 would lead to a preferential association with actin microfilaments, resulting in a more dynamic cytoskeleton. Dendritic maturation, which reflects a stabilization of microtubules, would be linked to a progressive reduction in the levels of both MAP-2c and phosphorylated adult MAP-2 (MAP-2a/b). The end of the period of synaptogenesis would

be then characterized by the adult cytoskeletal pattern, with abundant dephosphorylated MAP-2a/b. It has been postulated that excitatory synaptic activity, as well as other extracellular signals, influence the dendritic changes mentioned above mainly by differential effects on the phosphorylation state of MAP-2 (Aoki and Siekevitz, 1985) [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)].

MAP-2 and Synaptic Activity

MAP-2 is not only the prototypic dendritic protein, but also one of the most dynamic neuronal components; its levels and conformation are influenced by most neurotransmitter systems. MAP-2 changes following glutamate receptor activation are an integral part of the excitatory synaptic response, including classical models of plasticity such as LTP [reviewed by Johnson and Jope (Johnson and Jope, 1992)]. These synaptic-dependent changes in MAP-2 would be mediated by modifications of the balance between kinases and phosphatases. MAP-2 is a substrate of virtually all major neuronal kinases (Sanchez *et al.*, 2000). A particularly important link is the one with calcium-calmodulin-dependent protein kinase (CaMKII); the α subunit of this enzyme is a major constituent of the postsynaptic electrondense area present in dendritic shafts and spines (Strack *et al.*, 1997), a site where high mol. wt MAP-2 is also found in conjunction with actin. Because of its localization, and unique catalytic profile, CaMKII is a sensor of calcium entry secondary to glutamate receptor activation (Ghosh and Greenberg, 1995). CaMKII is not only influenced by glutamate receptors, but also modulates the density of glutamatergic synapses (Rongo and Kaplan, 1999). The close relationship between MAP-2 and CaMKII may be responsible for the role of CaMKII activity in limiting dendritic growth and promoting dendritic stabilization (Wu and Cline, 1998). The amino-terminal region of several MAPs, including MAP-2, also contains a binding domain for the regulatory domain (RII) of the cAMP-dependent protein kinase (PKA) [reviewed by Johnson and Jope and Sanchez *et al.* (Johnson and Jope, 1992; Sanchez *et al.*, 2000)]. Consequently PKA, which is part of the signaling pathway by which calcium influences gene expression, is anchored to the cell particulate fraction by MAP-2 and other A kinase anchoring proteins (AKAPs) (Mochly-Rosen and Gordon, 1998). Other kinases that phosphorylate MAP-2 include PKC, extracellular signal-regulated kinases, glycogen-synthase kinase 3, cyclin-dependent kinases, and microtubule-affinity-regulating kinases [reviewed by Sanchez *et al.* (Sanchez *et al.*, 2000)]. Much less is known about MAP-2 dephosphorylation by serine/threonine phosphatases. These enzymes, which are found in high concentrations in association with the neuronal cytoskeleton, seem to have relative substrate specificity (Sanchez *et al.*, 2000). For instance, PP2A dephosphorylates both MAP-5 and MAP-2 while PP2B appears to target only MAP-5 (Gong *et al.*, 2000).

Synaptic activity is critical in modulating developmental processes in the neocortex, including dendritic and synaptic formation (Greenough, 1984; Goodman and Shatz, 1993). Glutamate receptors appear to differentially affect the MAP-2 kinase/phosphatase balance and, in this way, microtubule stability. Neuronal depolarization induces a bimodal response with initial MAP-2 phosphorylation followed by late dephosphorylation (Quinlan and Halpain, 1996). This phenomenon would represent the sequential activation of the metabotropic and *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors. The former would activate kinases, such as CaMKII and PKC, whereas the latter would induce calcium-dependent

Table 1Semi-quantitative assessment of COX-2-ir and MAP-2-ir in RS and control neocortices^a

| Identification | Condition | Age (years) | Post-mortem interval (h) | Cause of death | COX-2-ir | | | MAP-2-ir ^b | | |
|----------------|-----------|-------------|--------------------------|------------------------|----------|-----|-----|-----------------------|-----|-----|
| | | | | | F | T | O | F | T | O |
| UMB52 | control | 5 | 19 | drowning | 2.5 | 3.0 | 2.0 | 3.0 | 2.0 | – |
| UMB24 | control | 10 | 17 | trauma | 2.5 | 2.5 | – | 3.0 | 2.5 | – |
| UMB26 | control | 18 | 17 | trauma | 3.0 | 2.5 | 3.0 | 2.0 | 2.0 | 2.0 |
| UMB175 | control | 20 | 21 | trauma | 3.0 | 2.5 | 3.0 | 2.5 | 2.0 | 2.0 |
| B2693 | RS | 5 | 31 | sudden | 1.0 | 0.5 | 2.0 | 2.0 | 2.0 | 0 |
| 93×87 | RS | 8 | 5 | intestinal obstruction | 0.5 | 1.0 | – | 2.5 | 2.5 | – |
| B2684 | RS | 8 | 12 | sudden | 2.5 | 3.0 | 3.0 | 1.0 | 1.0 | 1.5 |
| B1621 | RS | 10 | 5 | seizures | 2.0 | 1.0 | 3.0 | 1.0 | 1.0 | 1.0 |
| BRC630 | RS | 12 | 50 | sudden | 0.5 | 2.0 | 0.5 | 1.5 | 1.0 | 1.0 |
| B1616 | RS | 17 | 20 | seizures | 0 | 0 | 0.5 | 1.0 | 0 | 0 |
| B2897 | RS | 19 | 15 | seizures | 0.5 | 1.5 | 2.0 | 0.5 | 0.5 | 0 |
| B2018 | RS | 21 | 11 | seizures | 1.0 | 1.5 | 1.0 | 0.5 | 0.5 | 0.5 |
| B2575 | RS | 25 | 11 | seizures | 0.5 | 0.5 | 1.5 | 0.5 | 1.0 | 0 |
| 93×47 | RS | 31 | 20 | unknown | 2.0 | 1.0 | 3.0 | 2.0 | 1.0 | 0.5 |

COX-2, cyclooxygenase-2; ir, immunoreactivity; F, frontal; T, temporal; O, occipital. Reprinted with permission (Kaufmann *et al.*, 1997a).^aAdaptation of scoring system for gray matter neurons previously published (Evers and Uylings, 1994).^bPresented at the 1995 Neuroscience Meeting (Kaufmann and Naidu, 1995).

phosphatases (Quinlan and Halpain, 1996). Glutamate receptor activity might also modulate levels and turnover of MAP-2 (Woolf *et al.*, 1999; Sanchez *et al.*, 2000). The role of acetylcholine (ACh) in determining cortical connectivity is exemplified by its modulatory effect on ocular dominance patterns in visual areas (Bear and Singer, 1986). Cholinergic input was postulated to influence morphological neuronal maturation, after demonstration of close spatial and temporal relationships between ingrowing cholinergic afferents and differentiating cells (Hohmann and Ebner, 1985) and of nerve growth factor receptor expression by subplate neurons, the earliest neuronal elements to develop dendrites and synapses in the neocortex (Allendoerfer and Shatz, 1994). Experimental manipulations of cortical cholinergic fibers at birth in the mouse (Hohmann *et al.*, 1988) also directly demonstrate that ACh is critical for neurons undergoing dendritic differentiation. In this model, long-lasting changes in laminar organization and neuronal size (Hohmann *et al.*, 1988) are associated with reductions in dendritic arborizations (Hohmann *et al.*, 1991a). As in the adult rodent (Woolf, 1993), which shows selective reductions in MAP-2 immunostaining following acute cholinergic deficit, mice with early and transient disruption of cholinergic afferents also display a selective long-term decrease in MAP-2 expression (Kaufmann *et al.*, 1995a, 1997c). The functional significance of these changes is underscored by their association with aberrant patterns of thalamocortical connections (Hohmann *et al.*, 1991b) and with behavioral anomalies such as abnormal spontaneous activity and learning deficits (Bachman *et al.*, 1994). Finally, MAP-2 levels, conformation and turnover may be critical for learning paradigms of neocortical origin, as demonstrated for Pavlovian conditioning (Woolf *et al.*, 1994, 1999) and passive avoidance (Van der Zee *et al.*, 1994) in the rat brain.

Dendritic Proteins in Mental Retardation

Based on the fact that profiles of dendritic protein expression are related to specific stages of dendritic development (Matus *et al.*, 1986; Tucker, 1990; Johnson and Jope, 1992; Riederer *et al.*, 1995; Kaufmann *et al.*, 1997a), with 'adult' steady state levels that are related to both developmental and regeneration-induced expression (Ma *et al.*, 1999; Ramon-Cueto and Avila, 1999; Sanchez *et al.*, 2000), and that immunochemical detection of

these dendritic constituents is feasible in human post-mortem samples (Ang *et al.*, 1991; Allendoerfer and Shatz, 1994; Honig *et al.*, 1996; Kaufmann *et al.*, 1995b, 1997a,b), we have begun to characterize neocortical patterns of dendritic protein expression in two 'dendritic' disorders: RS and DS. RS is a developmental disorder that affects almost exclusively females, with a large proportion of cases linked to mutations in the X chromosome gene *MeCP2* (Amir *et al.*, 1999; Wan *et al.*, 1999). *MeCP2* is a transcriptional regulator that represses the transcription of methylated gene sequences (Stratling and Yu, 1999). RS is characterized by severe neurologic impairment, including MR, seizures and stereotypic movements (Naidu, 1997; Kaufmann and Moser, 2000). Neuroanatomically, RS shows generalized reduction in perikaryal size and dendritic arborizations with relative preservation of neuronal number and cortical lamination (Kaufmann *et al.*, 1998). DS, the most common genetic condition associated with MR (Moser, 1995), is characterized by abnormal physical and neurologic growth. In addition to malformations involving mainly the heart and gastrointestinal tract, brain growth is delayed in DS. Histologically, neocortical laminar formation is irregular, myelination of cortical fibers is delayed, and there is a reduction in dendritic length and spine dysgenesis (Wisniewski, 1990; Golden and Hyman, 1994; Wisniewski and Kida, 1994; Kaufmann and Moser, 2000).

By immunochemical methods, we have characterized the expression of proteins linked to early dendritic development (MAP-5 and MAP-2) and compared them with those associated with late phases of dendritic formation (NF-H) in RS and DS. In RS there is a selective reduction in MAP-2 immunostaining when compared with NF-H (Kaufmann *et al.*, 1995b, 1997a). Particularly severe was the decrease in MAP-2 immunoreactivity in superficial white matter neurons, which was not observed in DS (Kaufmann and Naidu, 1995; Kaufmann *et al.*, 1995b). Considering that neurons in the white matter are descendants of subplate neurons (Allendoerfer and Shatz, 1994), MAP-2 abnormalities in RS may reflect a disturbance in early cortical differentiation. Moreover, a decrease in MAP-2 immunostaining was a generalized phenomenon in RS since it was detected in multiple cortical regions (Table 1) (Kaufmann *et al.*, 1997a). Interestingly, cyclooxygenase-2 (an enzyme involved in synthesis of prostaglandins, which is activated as an early gene) (Kaufmann

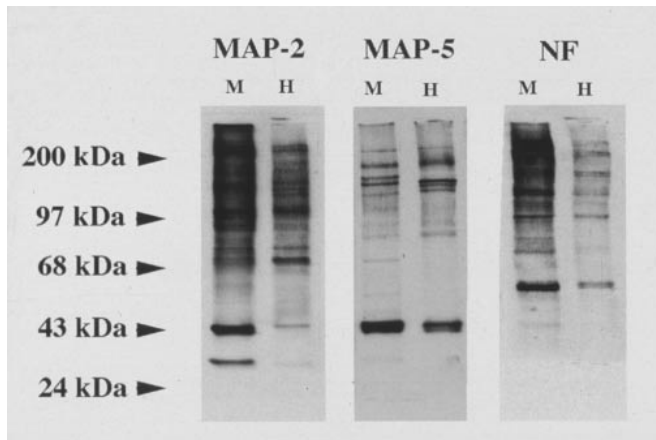


Figure 1. Immunoblotting patterns of MAP-2, MAP-5 and nonphosphorylated neurofilaments (NF) in cytosolic fractions from monkey (M) and human (H) neocortical gray matter homogenates. Reprinted with permission (Kaufmann *et al.*, 1997b).

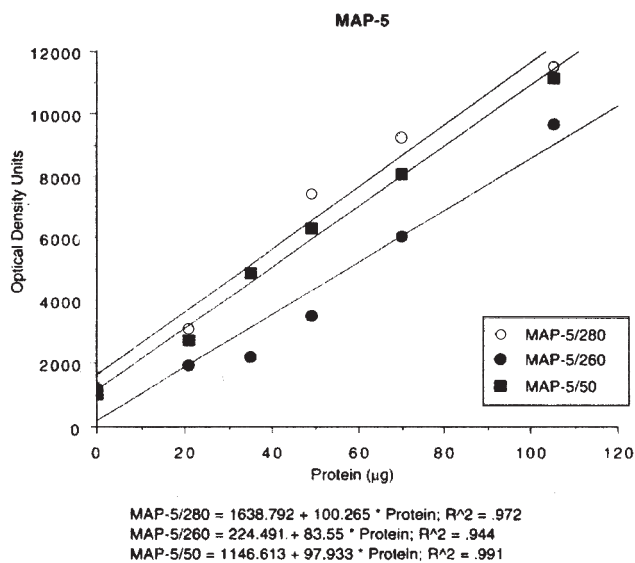


Figure 2. Tissue curve for MAP-5 immunoreactive bands, using increasing amounts of cortex homogenates. Represented are the high mol. wt MAP-5 doublet (260 and 280 kDa, separately) and the prominent 45 kDa band. Reprinted with permission (Kaufmann *et al.*, 1997b).

et al., 1996; Kaufmann and Worley, 1999) immunoreactivity was less reduced in the occipital cortex (Table 1) (Kaufmann *et al.*, 1997a), as expected from the Golgi impregnation studies (Armstrong *et al.*, 1995, 1998). Following the demonstration that cytoskeletal proteins are stable and quantifiable in post-mortem samples (Figures 1 and 2) (Kaufmann *et al.*, 1997b), quantitative immunoblotting showed that the selective reduction in MAP-2 immunostaining was due to reduced MAP-2 levels and not to changes in conformation (Table 2) (Kaufmann *et al.*, 1997c). Furthermore, MAP-5, another early dendritic protein, was also reduced whereas NF levels were unchanged (Table 2) (Kaufmann *et al.*, 1997c). In contrast, in DS there was a relative increase (per neuron) in all the examined proteins: MAP-5, MAP-2, NF-M and NF-H (Table 2) (Kaufmann *et al.*, 1997c). This increase is particularly significant considering that it is also present in children

Table 2

Summary of statistical comparisons between RS and normal (NC) and abnormal (DS) controls

| Epitope | Comparison | |
|-----------------------------|--------------|--------------|
| | RS versus NC | RS versus DS |
| High mol. wt MAP-2 | $P < 0.05$ | $P < 0.01$ |
| MAP-2 proteolytic fragments | $P < 0.01$ | $P < 0.01$ |
| 68 kDa MAP-2 | $P < 0.05$ | $P < 0.01$ |
| High mol. wt MAP-5 | NS | NS |
| 50 kDa MAP-5 | $P < 0.05$ | $P < 0.01$ |
| High mol. wt NF | NS | $P < 0.01$ |
| Medium mol. wt NF | NS | $P < 0.01$ |
| 45 kDa NF | NS | $P < 0.01$ |

NS, statistically not significant. Reprinted with permission (Kaufmann *et al.*, 1997c).

and adult subjects with DS, who show decreased dendritic arborizations (Takashima *et al.*, 1981; Becker *et al.*, 1986).

These immunochemical evaluations of RS neocortex suggested that dendritic length and dendritic cytoskeletal protein immunoreactivity were not in complete agreement. Simple reductions in dendritic arborizations should predict generalized decreases in cytoskeletal proteins, not differential changes such as those observed for MAP-2 or MAP-5. Moreover, assessments of DS frontal cortex by immunoblotting showed an increase in virtually all cytoskeletal proteins under evaluation. As these protein levels were calculated as ratios to the neuronal cytoplasmic marker neuron-specific enolase (NSE), in order to correct for differences in neuronal packing density, these DS data should be interpreted as a relative increase per neuron. Since there is a characteristic reduction in frontal cortex dendritic length, elevated levels of cytoskeletal proteins in DS most likely represent accumulation of these proteins in shorter dendrites. These findings indicate that overall assessments of immunostaining may not provide a complete picture of dendritic protein expression, and that a detailed analysis of immunohistochemical preparations may be necessary to interpret both immunohistochemical and immunoblotting data. The present study represents, to our knowledge, the first attempt to address this issue. We designed a semiquantitative evaluation of immunoreactivity, in immunohistochemical preparations, in order to determine whether there were specific neuronal populations or compartments contributing preferentially to these changes in RS. We focused on the pericentral, frontal motor or postcentral somatosensory cortex because these regions are major recipients of cholinergic afferents, a neurotransmitter system postulated as selectively affected in RS (Kaufmann *et al.*, 1998). We studied only pyramidal neurons since they represent the main neuronal type in the neocortex, and their MAP-2 expression is influenced by ACh and other neurotransmitters [reviewed by Johnson and Jope and Woolf (Johnson and Jope, 1992; Woolf, 1993)]. The selection of these cortical regions and neuronal populations would, therefore, potentially increase the possibility of finding cytoskeletal protein patterns that are specific and significant to RS pathogenesis. In order to accomplish these goals, we first improved upon previous protocols for cytoskeletal protein immunostaining by empirically assessing different antigen retrieval buffer solutions. These efforts led to a quality of immunostaining compatible not only with the cell population level of analysis, but also with layer and individual neuron-based evaluations. We then designed a scoring system that evaluates the density of immunolabeled cellular processes and intensity of immunostaining per neuronal soma or dendrite. These analyses

Table 3

Summary of subjects analyzed in this study

| Identification | Brain bank no. | Age (years) | Post-mortem interval (h) | Cause of death |
|----------------|----------------|-------------|--------------------------|--|
| Control 1 | UMB 52(M) | 4 | 19 | drowning |
| Control 2 | UMB 300 | 4 | 12 | congenital coronary artery malfunction |
| Control 3 | UMB 449 | 4 | 18 | trauma |
| Control 4 | UMB 269 | 5 | 36 | cardiac arrhythmia |
| Control 5 | UMB 163(M) | 6 | 21 | drowning |
| Control 6 | BTB 244 | 7 | 23 | trauma |
| Control 7 | UMB 24 | 9 | 17 | trauma |
| Control 8 | BTB 509 | 16 | 34 | trauma |
| Control 9 | UMB 72 | 18 | 29 | trauma |
| Control 10 | UMB 26 | 18 | 16 | trauma |
| Control 11 | UMB 55 | 19 | 17 | trauma |
| Control 12 | UMB175 | 20 | 21 | trauma |
| RS 1 | UMB 448 | 2 | 30 | RS complications |
| RS 2 | B2693 | 5 | 31 | sudden |
| RS 3 | 93×87 | 8 | 5 | intestinal obstruction |
| RS 4 | B2684 | 8 | 8 | sudden |
| RS 5 | BRC 630 | 10 | 48 | sudden |
| RS 6 | B1621 | 10 | 10 | seizures |
| RS 7 | B1616 | 17 | 17 | seizures |
| RS 8 | B2897 | 19 | 15 | seizures |
| RS 9 | B2018 | 22 | 21 | seizures |
| RS 10 | B2575 | 25 | 11–12 | seizures |
| RS 11 | 93×47 | 31 | <24 | unknown |

(M), male control.

disclosed layer-specific changes in dendritic protein immunostaining, which are described in detail in the following sections.

Materials and Methods

Tissue

Human cortical samples were obtained at autopsy and immersion-fixed in 10% buffered formalin for at least 2 weeks. They included 11 classical RS cases (age range in years, 2–31 years; median, 10 years) and 12 normal control subjects (10 female and two male; age range, 4–20 years; median, 9 years). Detailed information about the cases, and their corresponding controls, is provided in Table 3. The RS tissue was provided by Drs D. Armstrong (Texas Children's Hospital/Baylor College of Medicine) and S. Budden (Oregon Health Sciences University) and the Brain Tissue Resource Center at McLean Hospital/Harvard Medical School. Control samples were obtained from the University of Maryland Brain and Tissue Bank for Developmental Disorders. Use of this post-mortem material was approved by the Institutional Review Board for Human Subjects in Medical Research of the Johns Hopkins University School of Medicine. Cortical sampling included primary motor (area 4 of Brodmann) and somatosensory (areas 1, 2, 3 and 5 of Brodmann) regions. After fixation, the tissue was embedded in paraffin and sectioned at 10 μ m and processed for immunohistochemistry. Standard neuropathological evaluation of microscopic preparations by Dr Armstrong and one of the authors (W.E.K.) did not reveal any incidental abnormality [normal cortical lamination, small neuronal somas and more densely packed gray matter, as reviewed by Kaufmann *et al.* (Kaufmann *et al.*, 1998)] or obvious effect of post-mortem delay.

Immunohistochemistry

Sections were preincubated in 3% H₂O₂ and 0.3% Triton X-100 before microwaving for antigen retrieval. For the latter, we used a variation of the protocol we reported previously (Kaufmann *et al.*, 1997a), which was an adaptation of the method described by Evers and Uylings (Evers and Uylings, 1994a,b). For MAP-2 immunostaining, sections were microwaved in Tris buffer (pH 7.6) for 5 min twice. For neurofilament immunohistochemistry, tissues were microwaved at a lower pH (4.5) in citrate buffer for the same period of time. Following this, sections were blocked in 3% horse serum (Chemicon, Temecula, CA) and sub-

sequently incubated with primary antibody overnight at 4°C. Antibodies included the monoclonal SMI-52 (Sternberger, Baltimore, MD), directed against all molecular forms of MAP-2 (Figure 1) (Kaufmann *et al.*, 1997b), which was diluted at 1:500, and the monoclonal SMI-32 (Sternberger, Baltimore, MD), which recognizes predominantly the high mol. wt non-phosphorylated neurofilament and to a lesser extent the medium mol. wt neurofilament protein (Figure 1) (Kaufmann *et al.*, 1997b), diluted at 1:750. As negative controls, sections were incubated with standard mouse immunoglobulin G (Jackson Immuno Research, West Grove, PA) at comparable dilutions to those used for the primary antibodies.

Following incubation with the primary antibodies, staining was developed according to the avidin-biotin-peroxidase method using a Vectastain ABC-elite kit (Vector, Burlingame, CA). Briefly, sections were washed in phosphate-buffered saline, incubated with biotinylated anti-mouse secondary antibody, washed again in the same buffer and then incubated in avidin-biotin-peroxidase complex. Diaminobenzidine (DAB; Sigma, St Louis, MO) was used as the chromogen. The length of incubation in solution with DAB was determined by low power microscopic inspection. Slides were then washed, dehydrated and coverslipped using DPX (Sigma-Aldrich, St. Louis, MO). Both adjacent sections and same slides were either stained by the Nissl method or counterstained with hematoxylin for general histological orientation.

Semiquantitative Analysis

Pyramidal neurons of layers II–III and V–VI from optimally stained sections were analyzed blindly. We evaluated the intensity of cytoskeletal protein immunoreactivity of both neuronal cell bodies and dendrites. For this purpose, we expanded the global semiquantitative scoring system for the assessment of somatic/dendritic proteins we reported previously (Kaufmann *et al.*, 1997a). This new system focuses on intensity of immunolabeling in each major layer and neuronal compartment, which is analyzed separately. Evaluations are based on staining throughout the layer and consider the following parameters: number or density of labeled somas, number or density of immunostained dendritic profiles, intensity of soma immunostaining, and intensity of dendritic immunolabel. The scoring of density of immunoreactive processes reflects, in large extent, the intensity and distribution of the label in each neuronal compartment. The grading is as follows. For density of immunolabeled somas/dendrites: 0 = none or few; 1 = few scattered; 2 = many scattered; 3 = many densely packed. For intensity of somatic/dendritic immunostaining: 0 = no or minimal staining; 1 = weak staining; 2 = intermediate or mixed (weak and heavy) intensity of staining; 3 = intense staining.

All three authors participated in the design and implementation of the scoring system. After determining a high level of inter- and intra-rater agreement, all reported evaluations were carried out blindly by S.M.M.

Statistical Analyses

In order to analyze the semi-quantitative scores of immunoreactivity patterns displayed in Tables 4 and 5, we carried out first descriptive statistical tests. For this purpose, the scores expressed as a range from – to ++ were transformed to a scale ranging from 0 to 3. These analyses (not shown) demonstrated that, for most of the parameters, the data were not distributed normally but skewed towards higher or lower scores. The latter was particularly true for the RS group. Consequently, nonparametric tests, specifically the Wilcoxon–Mann–Whitney test for independent samples (Siegel and Castellan, 1988), were performed.

A second level of analysis consisted of converting the raw scores into categories of low and high level of immunoreactivity. For all parameters but dendritic layers II–III MAP-2 intensity and somatic layers II–III NF intensity, scores of 0 and 1 were labeled as low and 3 and 4 as high levels of immunoreactivity, respectively. The former variables were scored only as 0 and 1, which were categorized as low and high, respectively. These categorical data were analyzed by chi-square analyses. The results of these two types of analyses are depicted in Table 6 as *P* values.

In addition, the relationship between age and the different immunoreactivity measures was analyzed by nonparametric tests. Specifically, we used the Spearman rank-order correlation coefficient (Siegel and Castellan, 1988). These results are reported in the following section.

Table 4

Semiquantitative assessment of MAP-2 immunoreactivity in RS and control pericentral cortices

| Identification | Layers II–III | | | | Layers V–VI | | | |
|----------------|---------------|------|------|------|-------------|------|------|------|
| | (#S) | (SI) | (#D) | (DI) | (#S) | (SI) | (#D) | (DI) |
| Control 1 | + | ± | ± | – | + | ± | ± | + |
| Control 2 | + | – | + | – | + | – | + | ± |
| Control 3 | – | – | + | ± | ± | – | + | ± |
| Control 4 | + | – | – | – | + | ± | + | ± |
| Control 5 | + | – | + | – | + | + | + | ± |
| Control 6 | + | ± | ± | – | + | ± | + | ± |
| Control 7 | ± | – | ± | – | + | + | + | ± |
| Control 8 | + | – | + | ± | + | – | + | + |
| Control 9 | ± | – | ± | – | ± | ± | ± | ± |
| Control 10 | + | ± | + | ± | + | + | + | + |
| Control 11 | + | – | + | – | + | + | + | + |
| Control 12 | ± | ± | + | ± | + | ± | + | + |
| RS 1 | + | ± | + | ± | + | ± | ± | + |
| RS 2 | + | ± | – | – | + | ± | ± | – |
| RS 3 | ± | – | + | ± | + | ± | + | ± |
| RS 4 | ± | ± | – | – | + | ± | ± | ± |
| RS 5 | + | – | – | – | + | ± | – | + |
| RS 6 | + | – | – | – | + | ± | – | ± |
| RS 7 | + | – | ± | – | + | – | + | – |
| RS 8 | – | – | + | – | ± | – | + | ± |
| RS 9 | ± | – | + | ± | + | – | + | ± |
| RS 10 | ± | – | – | – | + | ± | – | – |
| RS 11 | + | ± | ± | – | + | ± | ± | ± |

The data present an adaptation of the scoring system for gray matter neurons previously published (Kaufmann *et al.*, 1997a). #S, number of somas; SI, soma intensity; #D, number of dendrites; DI, dendrite intensity.

Results

One of the most consistent anatomical features in MR is the presence of dendritic pathology in the cerebral cortex, including reductions in dendritic arborizations, and abnormal morphology and density of dendritic spines. One approach for further characterizing these dendritic abnormalities is the study of cytoskeletal proteins, constituents that play a role in dendritic formation and function, and are developmentally regulated. To date, the study of cytoskeletal protein expression in MR has been circumscribed to immunochemical methods applied predominantly to RS. These investigations have demonstrated that in RS there are selective reductions in MAP-2 immunostaining (Kaufmann *et al.*, 1995b, 1997a) and levels (Kaufmann *et al.*, 1997c). Topographic patterns of dendritic protein expression also seem to be protein-specific. In RS, decreased MAP-2 immunostaining involved all three frontal, temporal and occipital regions (Table 1) (Kaufmann *et al.*, 1997a). The latter area, which is relatively spared in terms of dendritic length, was at least as involved as the other cortices (Table 1) (Kaufmann *et al.*, 1997a). In contrast, cyclooxygenase-2 immunoreactivity was less reduced in the occipital cortex (Table 1) (Kaufmann *et al.*, 1997a) as expected from the dendritic labeling studies (Armstrong *et al.*, 1995, 1998). In addition, in our preliminary immunoblotting study, almost all cytoskeletal proteins under evaluation were increased in DS when compared with controls or RS (Table 2) (Kaufmann *et al.*, 1997c). Considering that in DS there is typically a decrease in dendritic length, these data suggested accumulation of MAP-2 and other cytoskeletal proteins in DS frontal cortex. Because of this lack of correlation between global evaluations of MAP-2 immunoreactivity and dendritic length in both RS and DS, we designed a semiquantitative scoring system for immunoreactivity. We intended to determine in immunohistochemical preparations whether there were specific neuronal populations (cortical layer) or compartments (soma versus dendrite)

Table 5

Semiquantitative assessment of neurofilament immunoreactivity in RS and control pericentral cortices

| Identification | Layers II–III | | | | Layers V–VI | | | |
|----------------|---------------|------|------|------|-------------|------|------|------|
| | (#S) | (SI) | (#D) | (DI) | (#S) | (SI) | (#D) | (DI) |
| Control 1 | + | – | ± | – | ± | + | ± | + |
| Control 2 | + | – | – | – | ± | + | ± | ± |
| Control 3 | + | – | ± | ± | + | + | + | + |
| Control 4 | ± | – | – | – | ± | + | + | + |
| Control 5 | + | ± | – | ± | + | + | ± | + |
| Control 6 | + | – | ± | – | + | + | + | + |
| Control 7 | + | – | ± | – | + | + | ± | ± |
| Control 8 | + | – | ± | – | + | + | + | + |
| Control 9 | + | ± | ± | ± | ± | + | + | + |
| Control 10 | + | + | ± | ± | ± | + | + | + |
| Control 11 | + | – | – | – | + | + | + | + |
| Control 12 | – | – | – | – | + | + | + | + |
| RS 1 | + | ± | ± | + | + | ± | ± | + |
| RS 2 | + | ± | ± | + | ± | + | ± | + |
| RS 3 | + | – | ± | – | + | – | + | ± |
| RS 4 | – | – | – | – | ± | – | – | – |
| RS 5 | ± | ± | + | ± | ± | + | + | + |
| RS 6 | + | ± | + | ± | + | ± | + | ± |
| RS 7 | ± | ± | ± | + | + | + | + | + |
| RS 8 | + | – | + | ± | + | + | + | + |
| RS 9 | ± | – | ± | ± | + | ± | + | + |
| RS 10 | + | ± | + | + | + | + | + | + |
| RS 11 | + | – | + | ± | + | ± | + | ± |

The data present an adaptation of the scoring system for gray matter neurons previously published (Kaufmann *et al.*, 1997a). #S, number of somas; SI, soma intensity; #D, number of dendrites; DI, dendrite intensity.

contributing preferentially to these changes in RS. We selected the somatosensory/motor cortex on the basis of its neurochemical and anatomical involvement, which is distinctive to RS (Kaufmann *et al.*, 1998). In order to accomplish these goals, we first modified our protocols for cytoskeletal protein immunostaining by empirically assessing antigen retrieval solutions that provide an increased signal to noise ratio. We then developed a highly reliable semiquantitative scoring system that evaluates the intensity of perikaryal and dendritic immunostaining, by estimating the number or density of immunolabeled somas and dendrites, and by evaluating the intensity of immunoreactivity in well-defined cellular processes. These analyses disclosed layer- and protein-specific changes in immunostaining, which are described in detail in the following paragraphs. Tables 4 and 5 summarize the scores for all subjects for MAP-2 and nonphosphorylated NF, respectively.

Patterns of MAP-2 and Nonphosphorylated Neurofilament Immunoreactivity

As previously described (Kaufmann *et al.*, 1995b), MAP-2 immunostaining in controls, as revealed by SMI-52, was relatively uniformly distributed throughout the cortex under study. Closer inspection showed that MAP-2 immunoreactive neurons corresponded exclusively to pyramidal neurons in layers II–III and V–VI, and spindle-shaped neurons in superficial white matter. Numerous dendrites, many of them ill-defined, were present in the background of all layers. Qualitative inspection also evidenced smaller cell bodies and a general reduction in immunostaining in RS, particularly in the lower layers (Figure 2, motor cortex).

Nonphosphorylated NF immunoreactivity was expressed in a more restricted fashion in normal controls. Staining was present in layers II–III and V–VI, but not in white matter. Contrasting with MAP-2, which labeled a large number of dendrites, many of which were not obviously connected with cell bodies, NF

Table 6

Summary of comparative statistical analyses of semiquantitative scores of MAP-2 and neurofilament immunoreactivity

| Measurement | MAP-2 | | | | Neurofilaments | | | |
|---------------|-------|-------|--------------|------------|----------------|-------|--------------|------------|
| | RS | NC | Mann-Whitney | Chi-square | RS | NC | Mann-Whitney | Chi-square |
| Layers II-III | | | | | | | | |
| #S | 1.636 | 1.750 | NS | NS | 1.727 | 1.833 | NS | NS |
| SI | 0.364 | 0.333 | NS | NS | 0.545 | 0.333 | NS | $P = 0.14$ |
| #D | 1.091 | 1.750 | $P = 0.16$ | NS | 1.364 | 0.417 | $P = 0.08$ | $P = 0.05$ |
| DI | 0.273 | 0.333 | NS | NS | 1.273 | 0.583 | $P = 0.01$ | $P = 0.05$ |
| Layers V-VI | | | | | | | | |
| #S | 2.091 | 2.417 | NS | NS | 2.000 | 1.750 | NS | NS |
| SI | 0.727 | 1.083 | NS | $P = 0.03$ | 1.364 | 2.333 | $P = 0.05$ | $P = 0.02$ |
| #D | 1.636 | 2.417 | $P = 0.09$ | $P = 0.04$ | 1.818 | 2.250 | NS | NS |
| DI | 0.909 | 1.500 | $P = 0.09$ | NS | 2.000 | 2.083 | NS | NS |

#S, number of somas; SI, soma intensity; #D, number of dendrites; DI, dendrite intensity; NS, statistically not significant.

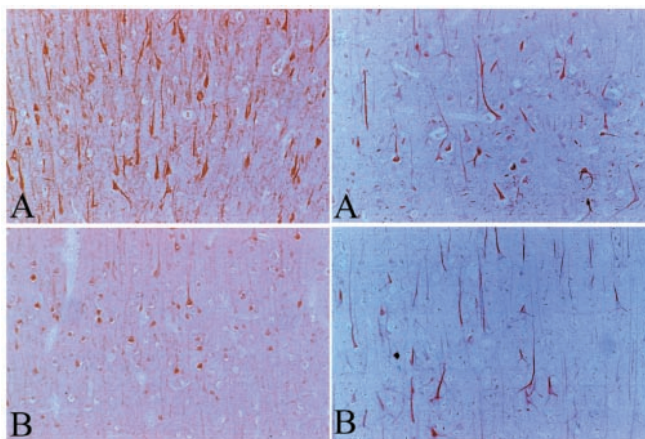
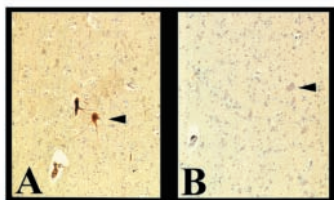
**Fig.3****Fig.4****Fig.5**

Figure 3. Immunohistochemical pattern of MAP-2 in motor cortex. (A) In control subjects, perikaryal and, predominantly, dendritic staining is seen throughout layer V. (B) In RS there is a marked reduction in MAP-2 staining involving both somas and dendrites in the same lamina. Note also the smaller cell bodies in RS. Both $\times 140$.

Figure 4. Pattern of nonphosphorylated neurofilament immunoreactivity in layer V-VI somatosensory cortex. (A) Control and (B) RS samples show comparable patterns of neurofilament immunostaining. Nonetheless, organization and prominence of dendritic profiles were decreased in RS. Both $\times 140$.

Figure 5. Dissociation between MAP-2 and neurofilament immunostaining. Consecutive sections of RS layer V motor cortex show that while (A) neurofilament immunoreactivity (arrowhead) was prominent, (B) MAP-2 immunostaining was markedly decreased. The arrowhead in (B) indicates a virtually negative perikaryon of a large pyramidal neuron. Both $\times 87$.

immunostaining outlined fewer either basal or apical dendrites, most of which were continuous with the somas. Moreover, cell bodies and fine dendritic processes were better delineated by this antibody. As we reported earlier, no obvious difference in intensity of label between control and RS subjects was appreciated (Figure 3, somatosensory cortex). Nevertheless, as for MAP-2, neuronal somas appeared smaller in the RS subjects.

Qualitative analyses also showed dissociation in the pattern of staining of MAP-2 and NF, particularly in layers V-VI. When consecutive sections were stained for each antibody, large pyramidal neurons were weakly immunoreactive for MAP-2 but strongly labeled by the NF antibody (Figure 4, motor cortex).

Layer-specific Changes in MAP-2 Immunostaining

Semiquantitative scoring of MAP-2 immunoreactivity (Table 4) showed that, for most parameters, scores were greater in layers V-VI in both groups. With the exception of intensity of staining in layers II-III somas, all parameters were reduced in the RS group. Wilcoxon-Mann-Whitney analyses showed that only three variables were decreased at the level of trend (Table 6): density of dendrites in layers II-III ($P = 0.16$) and density and intensity of dendrites in layers V-VI (both $P = 0.09$). When the raw score data were clustered as categories, the reduction in dendrite density in layers V-VI became significant ($P = 0.04$). Intensity of soma immunostaining in layers V-VI, which was not significant by nonparametric tests, also became significant at $P = 0.03$ (Table 6). Dendrite density in layers II-III and dendrite intensity in layers V-VI, which were decreased at a trend level by nonparametric tests, were not significantly reduced in the categorical analyses (Table 6).

In summary, the different measures of MAP-2 immunoreactivity showed more marked changes in the lower cortical layers. These reductions were statistically significant only when data were categorized as two distinctive levels of immunostaining, and involved both somatic and dendritic MAP-2 immunoreactivity in layers V-VI.

Laminar Pattern of Neurofilament Immunostaining

The semiquantitative scores of NF immunoreactivity (Table 5) contrasted with those of MAP-2 in that the scores were more similar in both strata. Moreover, there were changes in both directions in the RS group. Of the eight analyzed variables (four per layer), three were reduced in RS while four were increased. One parameter, intensity of dendritic staining in layers V-VI, appeared unchanged. Wilcoxon-Mann-Whitney tests showed two significant changes in the RS group: increase in dendritic intensity in layers II-III ($P = 0.01$) and reduction in somatic staining in layers V-VI ($P = 0.05$; Table 6). Density of dendrites in layers II-III was also elevated at the trend level ($P = 0.08$). Categorical analyses, by chi-square, were in agreement and significant for all the above-mentioned variables ($P = 0.02$ - 0.05 ; Table 6). They also showed that the increase in somatic staining in layers II-III in RS was at the trend level ($P = 0.14$; Table 6).

The data on NF immunoreactivity demonstrated more variable

and marked changes than those for MAP-2. In general, there was an increase in NF immunostaining in layers II-III and a reduction in somatic immunoreactivity in layers V-VI, similar to that found for MAP-2 in the same laminae.

Age-related Changes

Spearman rank-order correlation coefficient analyses were focused on the parameters that constituted trends or significant changes in the group comparison shown in Table 6. Only four of the eight analyzed variables demonstrated age-related changes. In terms of MAP-2, intensity of somatic and dendritic staining in layers V-VI increased with age in the control group ($P = 0.10$ and 0.01 , respectively), whereas they remained unchanged in RS. NF immunostaining scores were age-related only in layers II-III. Specifically, somatic staining increased with age in the control group ($P = 0.15$) while dendritic density increased with age in the RS subjects ($P = 0.11$). Again, these analyses showed the dichotomy of MAP-2 changes affecting predominantly lower layers whereas NF differences involved layers II-III.

Discussion

Reductions in dendritic length and abnormalities in morphology and density of dendritic spines are among characteristic anatomic features of MR-associated genetic syndromes (Huttenlocher, 1991; Kaufmann and Moser, 2000). Knowledge about the molecular bases of these structural changes is quite limited. A recent approach consists of the study of dendritic constituents by immunochemical methods, which obviate the post-mortem degradation of mRNA. These studies have focused on cytoskeletal proteins because of their abundance, stability and ontogenetic profiles that are related to their steady-state levels (Evers and Uylings, 1994a; Kaufmann *et al.*, 1997b; Ma *et al.*, 1999; Ramon-Cueto and Avila, 1999; Sanchez *et al.*, 2000). The most comprehensively characterized condition is RS, which displays a distinctive decrease in neuronal soma size and dendritic tree length (Kaufmann *et al.*, 1998). Immunocytochemical analyses that have assessed major cytoskeletal proteins have shown that there is a selective reduction in MAP-2 immunoreactivity when compared with nonphosphorylated NFs (Kaufmann *et al.*, 1995b, 1997a). These changes, initially evaluated in the motor/somatosensory cortical areas, seem to correspond to actual decreases in MAP-2 levels (Kaufmann *et al.*, 1997c). Measurements of cytoskeletal protein levels in cortex, by immunoblotting, provide a reliably quantitative estimate of the dendritic protein profile in RS and DS (Kaufmann *et al.*, 1997b,c). These analyses demonstrated that, while dendritic proteins linked to early stages of dendritic development – namely MAP-5 (the low mol. wt epitope) and MAP-2 – were significantly reduced in absolute and relative terms, NFs were virtually unchanged in RS (Kaufmann *et al.*, 1997c). Surprisingly, in DS all proteins but the high mol. wt MAP-5 were higher than in RS and, in many instances, higher than in controls (Kaufmann *et al.*, 1997c). Since these data corresponded to measurements of whole cortex homogenates, with corrections for changes in neuronal density by ratios to NSE, it can be concluded that immunoblotting assessments are difficult to interpret unless a link to anatomical features is established. Several obvious questions arise. How is it possible to find increases in dendritic protein levels per neuron in the presence of smaller neurons and/or shorter dendrites? Since cytoskeletal proteins are found in both somas and dendrites, do the selective reductions in MAPs affect somas and dendrites equally? Are there layer-specific changes? Although the global assessments of immunostaining,

based on approaches pioneered by Evers and Uylings (Evers and Uylings, 1994a,b), have shown that in RS the reduction in MAP-2 immunostaining is global, involving the frontal, parietal, temporal and occipital cortices (Kaufmann *et al.*, 1995b, 1997a), no detailed information about individual neuronal or layer features has been reported. For these reasons, we developed a semiquantitative scoring to assess cytoskeletal protein immunostaining in the somatic and dendritic compartments, and to evaluate laminar effects, relatively independent of neuronal size and dendritic length. Here we report on its first application to RS neocortex. We selected the precentral/postcentral gyri cortex because of its severe neurochemical and dendritic involvement in RS (Kaufmann *et al.*, 1998). The study attempted to further delineate the apparent dissociation in MAP-2 and nonphosphorylated NF immunoreactivity in RS neocortex, as a complement to the whole cortex measurements by immunoblotting.

Differential Neuronal and Laminar Changes in Cytoskeletal Protein Immunoreactivity in RS

Our qualitative observations confirmed our earlier reports demonstrating smaller cell bodies (Figures 2 and 3) and reduced MAP-2 immunostaining in RS (Figure 2). No clear changes in NF immunoreactivity were apparent (Figure 3). However, by serial staining we also found the expected (based on immunoblotting) dissociation in MAP-2 and NF immunoreactivities within the same neuronal population (Figure 4). Due to their larger size, these abnormalities were more obvious in layers V-VI pyramidal neurons. The semiquantitative evaluation, particularly in terms of intensity of somatic and dendritic immunostaining, was also easier in the lower layers because of their size and wider range of immunoreactivity (Tables 4 and 5). Nevertheless, several tendencies were evident. MAP-2 changes, which mainly consisted of decreased staining in RS, were more marked in layers V-VI while increased NF immunoreactivity seemed to be present in RS layers II-III.

In order to better delineate these patterns of cytoskeletal protein immunoreactivity, we analyzed statistically the raw scores depicted in Tables 4 and 5. These analyses, which were carried out using stringent criteria such as nonparametric tests, should nonetheless be taken with caution since the raw data are not 'in essence' quantitative. However, qualitative observations were corroborated, particularly by chi-square analyses that measured the frequency of cases with either low or high levels of immunoreactivity. Both somatic and dendritic (as density of labeled dendritic profiles) MAP-2 immunoreactivity in layers V-VI were significantly decreased (Table 6). Despite their larger variability, data on NF immunoreactivity demonstrated an increase in dendritic immunostaining in layers II-III. The latter pattern seemed similar in magnitude, but reversed in direction, to the one found for MAP-2 in the lower layers. Full score range and categorical comparisons showed other abnormalities in RS, which included a decrease in MAP-2 dendritic immunostaining in layers II-III and V-VI, an increase in somatic NF immunoreactivity in upper layers and a reduction in NF somatic immunostaining in lower layers (Table 6). Age-related increases in dendritic MAP-2 immunoreactivity in layers V-VI were also absent in RS. Other age-dependent changes, which constituted statistical trends, showed similar laminar distribution: MAP-2 in lower layers and NFs in upper layers. Our semiquantitative data could be summarized as follows: in RS there are reductions in MAP-2 expression in somas and dendrites in layers V-VI while there is an elevation in NF immunostaining in the upper cortical

layers and a decrease in dendrites of lower layers. These changes, especially the reductions in MAP-2, appear to represent a disruption in the normal ontogenetic trend.

Specificity of Cytoskeletal Protein Changes in RS

The original data presented here emphasizes the complexity of the changes in dendritic protein expression in RS neocortex. Whereas the selective reduction in MAP-2 immunoreactivity appeared to be a signature of this condition, its spatial distribution is not random, with greater involvement of the lower layers apparent. In terms of the neuronal compartment, this study shows that global cytoskeletal protein assessments also detect changes in the neuronal soma. In fact, it appears that in RS there is global down-regulation of cytoskeletal proteins in the cell bodies. The present analysis was also informative in demonstrating that there are concomitant increases in dendritic protein expression, which were circumscribed to NFs in the upper layers. The latter results raise the possibility of compensatory changes in the dendritic pathology associated with RS. Differential involvement of upper layers (involved in cortico-cortical circuits) and lower strata (mainly subcortical projecting neurons) in RS may also have repercussions on cortical physiology.

These protein-, compartment- and layer-specific changes in RS contrast with the quantitative analyses of dendrites by Golgi impregnations. Armstrong and colleagues (1995, 1998) have shown that in the frontal cortex both apical and basal dendrites in layers III and V are significantly shorter or less complex when compared with control and DS subjects. These discrepancies were anticipated by our previous study examining the topographic distribution of changes in MAP-2 and another dendritic protein, cyclooxygenase-2 (Table 1). MAP-2 immunoreactivity was decreased throughout the cortex, while COX-2 expression was relatively preserved in occipital cortex in correspondence with the dendrite labeling data (Table 1) (Kaufmann *et al.*, 1997a).

We conclude that semiquantitative evaluations as the one presented here provide valuable complementary data to morphologic and other, more global, immunochemical assessments. Despite this, several issues need to be considered. First, these studies require the development of appropriate immunohistochemical protocols to enhance staining in long-term fixed post-mortem samples. Other investigators have developed similar methods for the study of these tissues (Evers and Uylings, 1997). A second issue is the development of reliable scoring systems. Although our approach was highly consistent, it was not exempt of subjectivity and seemed to be less sensitive for changes affecting smaller neurons. For this reason, we introduced our statistical analyses as a way to disclose patterns of immunostaining rather than to provide precise or absolute measurements. These immunohistochemical evaluations should be seen as a demonstration of the need to complement traditional histological approaches with immunochemical analyses, as a link between structural techniques and quantitative molecular approaches (e.g. Northern or Western blotting), and as a guidance for the planning of investigations on the pathogenesis of cortical abnormalities in MR (e.g. animal models).

Selective Changes in Dendritic Proteins in RS: Pathogenetic Considerations

Until the recent demonstration of the link between mutations of *MeCP2* and the classical RS phenotype (Amir *et al.*, 1999; Wan *et al.*, 1999), most of the hypotheses regarding the RS neurologic

phenotype were based on neurochemical and neuroanatomical post-mortem studies. Monoamines, particularly deficits in ACh, had been implicated early in this condition (Kaufmann *et al.*, 1998). Our preliminary analyses of a mouse model of early but transient cholinergic deficit (Hohmann *et al.*, 1988), which shows reductions in dendritic arborizations (Hohmann *et al.*, 1991a), indicated a long-term reduction in MAP-2 levels by measurements similar to those reported for RS and DS (Kaufmann *et al.*, 1997c). Global assessments of immunoreactivity also demonstrated a selective reduction in MAP-2, when compared with NFs, months after the cholinergic deficit (Kaufmann *et al.*, 1995a). Therefore, the pattern of cytoskeletal protein expression in RS reported previously (Kaufmann *et al.*, 1995b, 1997a,c) and extended here could be, at least in part, the consequence of an early cholinergic deficit. The latter should be further examined by applying the semiquantitative scoring system to this mouse model. As MAP-2 is a protein whose ontogeny parallels dendritic growth [reviewed by Johnson and Jope and Sanchez *et al.* (Johnson and Jope, 1992; Sanchez *et al.*, 2000)] and as non-phosphorylated NF, particularly the high mol. wt variant, is expressed during late dendritic development (Shaw and Weber, 1982; Kaufmann *et al.*, 1997a), our finding of an age-independent reduction in MAP-2 in layers V–VI suggests that these cells were disturbed during their early dendritic formation. In support of this hypothesis, we have previously shown that there is an extreme reduction in MAP-2 immunoreactivity in white matter neurons in RS, in contrast with control and DS subjects (Kaufmann and Naidu, 1995; Kaufmann *et al.*, 1995b). As these neurons are subplate descendents and immediately precede the development of the lower cortical layers (Allendoerfer and Shatz, 1994), our latest findings suggest a protracted abnormality in MAP-2 expression during early cortical histogenesis. Delay or deficit in cholinergic afferents to the subplate may be a factor leading to disrupted neuronal differentiation of these cortical strata. Nevertheless, MAP-2 is a highly dynamic molecule whose expression is regulated by several neurotransmitter systems in the mature cortex (Johnson and Jope, 1992; Woolf, 1993; Van der Zee *et al.*, 1994). Recently reported glutamate receptor abnormalities, particularly those affecting the NMDA subtype, in RS frontal cortex (Blue *et al.*, 1999) may also play a role in these cytoskeletal protein changes. Our finding of increased NF immunostaining in layers II–III is intriguing. Although NFs are more stable proteins than MAP-2, NF-H levels (NF predominantly detected in this study) are increased by neurite membrane stimulation (Riederer, 1992). Since NF levels seem to modulate neurite caliber (Hoffman *et al.*, 1988), compensatory plastic phenomena involving NF expression in layers II–III should not be excluded. We have demonstrated in Huntington disease, by quantitative Golgi analyses, that layer III pyramidal neurons have increased dendritic length, suggesting plasticity by residual frontal neurons (Sotrel *et al.*, 1993).

Implications for Other Mental Retardation-associated Disorders and Future Directions

To date no primary defect in neuronal cytoskeletal protein expression has been reported in MR-associated disorders. However, several links between cytoskeletal proteins and their regulatory enzymes and MR have appeared. In addition to the previously mentioned potential roles in MR pathogenesis of dystrophin in Duchenne muscular dystrophy (Torelli *et al.*, 1992), LIMK1 in Williams syndrome (Monaco, 1996) and PAK3 in nonsyndromic X-linked MR (Allen *et al.*, 1998), X-linked Opitz syndrome, a disorder characterized by midline brain and somatic

malformations, may represent the first MAP mutation associated with MR. The defective gene in this condition, *MIDI1*, codes for a MAP (Quaderi *et al.*, 1997) that when carrying mutations such as those described in Opitz syndrome becomes incapable of associating with microtubules *in vitro* (Schweiger *et al.*, 1999).

Nevertheless, it is the study of cytoskeletal and cytoskeleton-related proteins as markers of developmental processes that has the greatest potential of disclosing mechanisms underlying neuronal and synaptic abnormalities in MR. Our preliminary work in RS and DS ought to be extended to other disorders, such as FraX, including their related animal models (Comery *et al.*, 1997). Even the data on DS, the most prevalent genetic disorder associated with MR (Moser, 1995), are quite limited. Most of the studies of synaptic pathology in DS have focused on presynaptic elements, particularly the synaptic vesicle-associated protein synaptophysin (Dietzmann and von Bossanyi, 1994; Wisniewski and Kida, 1994). Unquestionably, these data are important for understanding the relationship between both synaptic compartments. Despite the intrinsic limitations of the use of post-mortem samples, comprehensive analyses of MAP-2 and NFs, such as those reviewed here, have shown that neuronal and dendritic morphological evaluations underestimate the complexity of the molecular events underlying synaptic pathology. The novel semiquantitative analysis presented in this study provides a link between traditional anatomical methods and molecular approaches.

Our finding of potential molecular compensatory responses in certain cortical layers in RS underscores the relevance of these studies. First, a substantial portion of dendritic and synaptic development in humans occurs postnatally (Huttenlocher, 1999). This brings the prospect of interventions aiming at reversing, at least to some extent, disruptions of synaptic development. Recent evidence that growth factors, such as neurotrophins, are able to modulate dendritic formation in a layer-specific fashion (McAllister *et al.*, 1997) raises the possibility of a therapeutic strategy for dendritic disorders. Furthermore, the growing body of data on local protein synthesis in dendrites (Steward, 1995; Crino and Eberwine, 1996) supports the notion of devising specific schemes for modifying the molecular make up of dendrites. It is necessary to emphasize that among the proteins whose synthesis may occur at dendrites are such important regulators as MAP-2, the α -subunit of CaMKII and brain-derived neurotrophic factor. In fact, in FraX, the second most common MR-associated genetic disorder (Moser, 1995), there is a deficit in a protein (fragile-X mental retardation protein, FMRP) that shuttles transcripts between nuclear and cytoplasmic compartments (Feng *et al.*, 1997; Kaufmann *et al.*, 1999). FMRP is also localized to dendrites and dendritic spines (Feng *et al.*, 1997; Tamanini *et al.*, 1997). Advances in the neurobiology of FraX might, certainly, provide information about the general mechanism of dendritic plasticity that can be applied to other dendritic disorders. A final promising note, on the strategy for studying neuronal cytoskeletal proteins, is our recent data on olfactory neuroblast gene expression in FraX (Abrams *et al.*, 1999). By culturing olfactory mucosa receptor cells, we have been able to demonstrate similarities between FMRP patterns in lymphocytes and in these neurons (Abrams *et al.*, 1999; Kaufmann *et al.*, 1999). Analyses of olfactory neuroblasts may constitute valuable complements to post-mortem data, allowing us not only to study MR pathogenesis but also to monitor dendritic development and its changes *in vivo*.

Notes

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