4.2 Localization of RIM interacting proteins

Through their multiple protein domains RIMs have been shown in vitro to interact with multiple presynaptic proteins. However, for many of these binding partners it is not known to what degree their regional and cellular expression overlap throughout the brain. Because of their importance for the structural organization of the active zone and the Ca\(^{2+}\)-sensing step, distribution of the RIM interacting proteins Liprins, RIM-BPs and Synaptotagmins were analyzed.

4.2.1 Differential expression of the Liprin-\(\alpha\) protein family

To obtain information on the mRNA distribution of the Liprin-\(\alpha\) protein family, \textit{in situ} hybridizations were performed on sagittal and horizontal brain slices of adult (p28) and newborn (p0) rats with radioactive probes detecting Liprin1\(\alpha\), -2\(\alpha\), -3\(\alpha\) and -4\(\alpha\) (Figure 4.12). All four family members are expressed throughout the newborn brain in overlapping patterns that vary in different brain regions between the four isoforms (Figure 4.12 left row). While Liprin4\(\alpha\) is equally expressed in most brain regions, with the strongest expression levels in the hippocampus, lower cortical layers and olfactory bulb granule cells, the remaining isoforms show stronger regional differences. Liprin3\(\alpha\) mRNA is most abundant in the hind- and midbrain, including the thalamus, as well as in the hippocampus, while the striatum, subthalamic regions and the olfactory bulb show less expression. In the cortex, the outer cortical layers exhibit the strongest signal, showing an inverse staining compared to Liprin4\(\alpha\). Expression of Liprin2\(\alpha\), and to a lower degree of Liprin1\(\alpha\), is enriched in the forebrain, with the most intense labeling in the outer cortical layers, the hippocampus, the olfactory bulb and the striatum, while hind brain and brain stem show less expression.
Figure 4.12: Distribution of Liprins-α in the neonatal and adult brain. X-ray pictures of in situ hybridizations of the Liprin protein family on sagittal (p0, p28) and horizontal (p28) rat brains.
In the adult brain, Liprin1α and -4α expression declines, within most brain regions, except the cerebellum, the olfactory bulb and the hippocampus, showing only residual expression (Figure 4.12, middle and right column). In the hippocampus, Liprin1α is most strongly expressed in the gyrus dentatus granule cells, while Liprin4α can be detected in higher levels in the CA3 (ventral HC) and CA1 (dorsal HC) regions. Liprin2α mRNA is found at high levels throughout the adult brain, in particular in the olfactory bulb granule cells and mitral cells, cortical layer 2, the hippocampus and the cerebellum. In situ hybridization also revealed strong expression of Liprin3α, however the mRNA distribution varies between the different brain regions. The strongest signals were observed in the olfactory bulb, the cortex and the hippocampus. Compared to Liprin2α, the distribution of Liprin3α throughout the cortical layers is more uniform. Only weak signals were detected in striatum, thalamus, hindbrain and brainstem.
4.2.2 Synaptotagmin gene expression in the rat brain

Synaptotagmins are a large family of membrane trafficking proteins. They are evolutionary conserved and have 15 members in rodents and humans. The regional distribution of some of the Synaptotagmin isoforms has been published already (Berton et al. 1997, Ullrich & Sudhof 1995), but a comparative study of all Synaptotagmins is still missing. Therefore a systematic analysis of expression using radioactive in situ hybridization and quantitative real time RT-PCR as well as multiplex RT-PCR on a single cell level was performed.

4.2.2.1 Expression patterns of Synaptotagmin genes

The spatial expression pattern of Synaptotagmins-1 to -13 was analyzed in brains of rats (P28) by radioactive ISH and quantitative real time RT-PCR. To compare the expression of the different genes in different regions of the brain the ISH analysis was performed on horizontal, sagittal, and coronal sections. The ISH analysis allowed the reliable comparison of expression strength of individual isoforms between different brain regions (Table 4.3). However, due to variations in the sensitivity between different probes, a comparative analysis between the different isoforms can only be qualitative. Therefore, the relative expression levels of the different Synaptotagmins in the olfactory bulb, basal ganglia, cerebral cortex, hippocampus, diencephalon and cerebellum was additionally determined by quantitative real time RT-PCR (q-rtPCR) (Figure 4.13). For the individual Synaptotagmin isoforms the expression strength in the analyzed brain regions determined by q-rtPCR reflected the results obtained by ISH (Figure 4.13 and Figure 4.15, Table 4.3). The q-rtPCR revealed that Syt-1 is the most abundant Synaptotagmin isoform in all brain regions tested, whereas most of the other isoforms are only present at significantly lower levels. However, in some areas like the cerebellum other isoforms, i.e. Syt-2 and -12, were also expressed strongly. Expression levels for Syt-8 were below the detection limits of the ISH (data not shown) and barely detectable by q-rtPCR (Figure 4.14).
Table 4.3: Expression of Synaptotagmin-1 to -13 in various brain regions

X-ray films exposed for 1-2 weeks (grey background) and 4-6 weeks (white) due to general differences in the expression levels. Brain regions and abbreviations depicted on the left, mRNA labeling intensity on the right ranging from no labeling (-) to very strong labeling (++++) or strong punctate labeling (o).

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Figure 4.13: Synaptotagmin mRNA expression in various brain regions.
Quantitative real-time RT PCR Data of Synaptotagmin 1 - 13 from six different rat brain regions. Expression levels are normalized to internal Synaptophysin control. The 20% line is accentuated to ease comparison of different-scaled graphs.
N = four adult male rats; OB olfactory bulb; BG basal ganglia; CX cortex; HC hippocampus; DC diencephalon; CB cerebellum.
4 Results

Figure 4.14: Quantitative real-time RT PCR Data of Synaptotagmin 8.
Data from six different rat brain regions, generated with fluorescent probes. Expression levels are normalized to internal Synaptophysin control. N = four adult male rats; OB olfactory bulb; BG basal ganglia; CX cortex; HC hippocampus; DC diencephalon; CB cerebellum.

Figure 4.15: Distribution of Synaptotagmin genes in P28 rat brain.
X-ray film images showing the localization of all members of the Synaptotagmin gene family on horizontal sections of rat brain detected with radioactive in situ hybridization (ISH) using 35S labeled oligonucleotides specific for Synaptotagmins (Syts) -1 to -13 (Syt-8 was not detectable in P28 rat brain). Negative controls with excess unlabeled oligonucleotides were devoid of signal (Figure 4.16). BG basal ganglia; CB cerebellum; CO colliculus; CX cortex; HC hippocampus; MHb medial habenular nucleus; OB olfactory bulb; TH thalamus; WM white matter regions, i.e. corpus callosum, capsule, fimbria of the hippocampus.
4.2.2.2 Olfactory bulb

In juvenile rats, expression of eleven Synaptotagmins (Syt-1, Syt-3 – Syt-7, Syt-9 – Syt-13) was detected in the olfactory bulb in variable patterns; only transcripts for Syt-2 and Syt-8 were absent (Figure 4.13, Figure 4.15, Figure 4.17 and Table 4.3). Whereas Syt-9 mRNA was only found in the glomerular (Gl) layer and in mitral cells (Mi), transcripts for the remaining Synaptotagmin genes were also present in the granular layer (GrO). The most abundant Synaptotagmin isoform in the olfactory bulb is Syt-1, which showed its strongest expression in the mitral cell layer, and was equally expressed at high levels in the glomerular layer and the olfactory bulb granular layer. Labeling for Syt-3 and Syt-12 was detected in the three layers at equal intensity; however, their level of hybridization was overall low. Syt-6 and -10, and to a lower degree Syt-4 and -5 were predominantly expressed in the olfactory bulb granule cells and mitral cells. Syt-10 is only expressed at very low levels throughout the brain, but transcript levels...
in the granule cells are very high, thereby representing the only cell type with significant Syt-10 expression under native conditions. Syt-6 exhibited a similar pattern of expression in the olfactory bulb, even though Syt-6 transcript levels in most remaining parts of the brain are higher than the ones observed for Syt-10. Syt-7 and Syt-13 are most prominently expressed in the glomerular layer and the mitral cell layer, but at weaker levels also in the granular layer.

Figure 4.17: Synaptotagmin gene expression in the olfactory bulb of rat brain. Dark field images of emulsion-dipped tissue sections reveal the expression of Syts within the different cell layers of the olfactory bulb. EPI external plexiform layer; GI glomerular layer; GrO granule cell layer of the olfactory bulb; Mi mitral cell layer.

4.2.2.3 Cerebral cortex

In the cerebral cortex, ISH experiments revealed the expression of the mRNAs encoding 11 of the 13 Synaptotagmin genes examined in this study (Table 4.3, Figure 4.13, Figure 4.15 and Figure 4.18). Syt-8 and Syt-9 were not detectable in cortical cells by ISH and only very low Syt-9 mRNA levels were measured by quantitative real time RT-PCR. Whereas Syt-1, -3, -4, -5, -11, -12, and -13 were expressed in cortical layers II to VI of the frontal cortex as well as the motor cortex, Syt-2, -6, -7, and -10 exhibit a more layer-specific expression pattern. Syt-1 was very strongly and uniformly expressed throughout the cortex besides layer I. Syt-3, -5, -11 and -13 exhibited a similar more less uniform pattern of expression in the cortex, however, with the exception of Syt-11, their transcripts were
present at a lower level in all laminas examined. In particular, signals for Syt-3 and -13 were very weak. Syt-2 showed a distinct punctate labeling most likely reflecting expression in the big pyramidal neurons in lamina III and V. Expression of Syt-6 was strictly confined to layer V resulting in a low overall expression level as determined by quantitative real time RT-PCR. Syt-7 was predominantly found in neurons of layers V and VI, and at weaker levels in the upper laminas. Transcript levels of Syt-10 in the cortex were very low and were mainly restricted to layer II, V and VI. Syt-11, the one Synaptotagmin with a distinct expression in nonneuronal parts of the brain, was also detected in layer I and the cortical white matter regions like the corpus callosum, the external capsule and the fimbria of the hippocampus (white matter, WM).

Figure 4.18: Synaptotagmin gene expression in the cortex of rat brain. Dark field images of emulsion-dipped tissue sections reveal the expression of Syts within the different cortical layers.

4.2.2.4 Hippocampus

In the hippocampal formation, weak to strong expression or punctate labeling, indicating single cell expression in a small subset of cells was found in at least one of the two parts of the cornu ammonis pyramidal cell layers (CA1, CA3) and the dentate gyrus (DG) for most of the Synaptotagmin genes. The only exceptions were Syt-2, Syt-8 and Syt-9, for which almost no labeling could be de-
Results detected (Table 4.3, Figure 4.13, Figure 4.15 and Figure 4.19). However, analysis of dark field images of emulsion dipped tissue sections revealed that Syt-2 is exclusively found in a subpopulation of cells scattered mainly in the CA1 and CA3 subfields. Syt-1 mRNA was present at very high levels in the pyramidal cell layer of the CA subfields and the granular cell layer of the dentate gyrus. In addition, a strong punctuate labeling was found in the Hilus and to a lesser extent in the stratum radiatum and the stratum oriens, indicating that Syt-1 is expressed by the interneurons and/or glial cells located in these areas as well.

Figure 4.19: Synaptotagmin gene expression in the hippocampus. Dark field images of emulsion-dipped tissue sections reveal the expression of Syts within the different hippocampal subregions. CA cornu ammonis region; DG dentate gyrus; Hi hilus region; Sr stratum radiatum; scale bars = 500µm.

Syt-3, -4, -5, and -11 exhibited a similar more less uniform expression pattern with punctate single cell labeling in the hilar region. Their overall level of ex-
pression was weaker though, with the exception of Syt-11, which showed equally stronger expression in all subfields. Syt-7 was very prominently expressed in the DG, with lower, but still strong expression in the CA3 region and moderate levels in CA1. Several other Synaptotagmin isoforms showed even stronger subregion-specific variations in their expression patterns. Syt-10 transcripts were mostly present in the DG, even though the overall level of Syt-10 mRNA in the hippocampus was very low. Syt-6 exhibited a similar pattern of distribution, but furthermore showed intense labeling in a distinct subset of cells within the dentate gyrus and the CA subfields. In contrast, strong expression of Syt-12 was only observed in the CA1 region. Syt-13 mRNA was moderately expressed in the CA3 layer, whereas its expression in the other parts of the hippocampus was relatively weak.

4.2.2.5 Basal ganglia

Within the main structures of the basal ganglia, such as caudate putamen (CPu), ventral pallidum (VP), and nucleus accumbens (Acb), radioactive ISH revealed the presence of all Synaptotagmin isoforms, albeit with diverging distributions (Table 4.3, Figure 4.13, Figure 4.15 and Figure 4.20). Transcripts for Syt-1, Syt-3 – Syt-7, Syt-11 and Syt-13 were detected in the putamen and nucleus accumbens in an abundance that was similar or lower than the one observed in the cortex. Syt-2 showed a strong punctate labeling in the ventral pallidum (VP) and the islands of calleja (ICj), whereas the hybridization pattern in the putamen was weaker and more uniform. In contrast, expression of Syt-6 was confined to the putamen, nucleus accumbens and the islands of calleja as the ventral pallidum and the lateral septal nucleus were void of a hybridization signal. Interestingly, in the ISH Syt-9 and Syt-12 were the only Synaptotagmin isoforms with a higher abundance in the putamen than in the cortex (Table 4.3 and Figure 4.13), even though the overall level of expression was low to moderate in the basal ganglia. Labeling for Syt-10 can only be detected in the ventral pallidum.

4.2.2.6 Midbrain and Diencephalon

In the midbrain and the diencephalon, the level of expression of the individual Synaptotagmin genes varied from very strong, as observed for Syt-1 and Syt-11, to not detectable in Syt-10 (Table 4.3, Figure 4.13, Figure 4.15 and Figure
Syt-1 was expressed in all parts of the diencephalon, most prominently in the thalamus (TH), the medial amygdaloidal nuclei (MeA) and the superficial layers of the superior colliculi (SC). Strong expression of Syt-1 mRNA was also detected in the inferior colliculi (IC), the anterior pretectal nucleus (APT), the hypothalamic nuclei (HY) and zona incerta (ZI). Interestingly, the substantia nigra (SN) only showed weak Syt-1 expression.

Figure 4.20: Sagittal view of Synaptotagmin gene expression in rat brain. Inverted scans of films exposed to labeled tissue sections reveal the expression of Syts within the basal ganglia, midbrain, diencephalon and brainstem. Acb nucleus accumbens; APT anterior pretectal nucleus; AT anterior thalamus; CPU caudate putamen; DG dentate gyrus; DT dorsal thalamus; HY hypothalamic nuclei; IC inferior colliculus; ICj Islands of Calleja; MeA medial amygdaloidal nucleus; MeAD medial amygdaloidal nucleus, anterio-dorsal part; Pn pontine nuclei; PT posterior thalamus; Rt reticular thalamic nucleus; SC superior colliculus; SN substantia nigra; TH thalamus; VP ventral Pallidum; VT ventral thalamus; ZI zona incerta.

Syt-2 exhibited a more restricted pattern of expression. Highest levels of expression were found in the inferior colliculus and zona incerta. Distinct parts of the anterior and ventral thalamus as well as the reticular thalamic nucleus (Rt),
the substantia nigra, the anterior pretectal nucleus and the superior colliculi showed moderate to strong labeling, too. Interestingly, the dorsal and posterior parts of the thalamus were void of any signal, while the anterior, ventral and ventro-lateral nuclei were strongly labeled. Syt-3 and -4 were found throughout the diencephalon in a more less uniform distribution, at an overall moderate level of expression. However, Syt-4 showed stronger expression in the medial amygdala, especially the anterior-dorsal amygdaloidal nucleus (MeAD). Syt-5 expression was lower in most parts of the midbrain, only the posterior thalamus (PT), hypothalamic nuclei (HY) and the inferior colliculus showed moderate signals. Interestingly, labeling in the medial amygdaloidal nuclei was very strong, while the substantia nigra did not show Syt-5 expression. Moderate Syt-6 signals were confined to the reticular thalamic nucleus (Rt), the APT, the inferior colliculus (IC) and, to a lesser extent, the superior colliculus (SC), while the thalamus and substantia nigra (SN) show no Syt-6 expression. In contrast, the thalamus was the midbrain region with the highest level of expression for Syt-7 and Syt-9. However, whereas Syt-7 was one of the most abundant Synaptotagmin isoforms in the thalamus besides Syt-1 and Syt-11, the overall expression level of Syt-9 was low. Moderate Syt-7 mRNA labeling was found in the dorsal collicular layers, where also weak labeling for Syt-9 was detected. Strong Syt-9 expression was only found in the medial habenular nucleus (MHb, Figure 4.15). Syt-11 was very strongly expressed throughout all parts of the diencephalon and mesencephalon. Syt-12 labeling was uniformly low, with strongest levels in the APT, the reticular thalamic nucleus (Rt) and subthalamic regions, for example the amygdala and zona incerta. The thalamus only showed a weak expression, and also the substantia nigra expressed low levels of Syt-12. Syt-13 mRNA was found in the anterior pretectal nuclei and the anterior-dorsal thalamus, while the posterior thalamus exhibited weaker expression.

4.2.2.7 Brainstem

In the brainstem, transcripts of all Synaptotagmin isoforms, except Syt-9, could be detected (Table 4.3 and Figure 4.20). However, the overall level of expression was quite low, only Syt-2 and Syt-11 showed a strong labeling. Syt-1, the isoform with the highest abundance in the brain besides Syt-11, was less abundant in the brainstem than in all other brain regions. Only in the pontine nuclei a
moderate hybridization signal could be detected. On the other hand, Syt-2 is most strongly expressed in the hindbrain, in particular in the brain stem, where a strong punctate distinct labeling for Syt-2 was observed. Syt-11 is the most abundant Synaptotagmin isoform in the brain stem as its mRNA is uniformly expressed and supposedly present in spinal neurons and glial cells.

### 4.2.2.8 Cerebellum

Figure 4.21: Synaptotagmin gene expression in the rat cerebellum. Dark field images of emulsion-dipped tissue sections reveal the expression of Syts within the different hippocampal subregions. cbw cerebellar white matter; GC cerebellar granule cells; ML molecular layer; PC purkinje cells; scale bars = 100µm.

In the cerebellar granule cell layer (GC), medium to strong expression of most Synaptotagmin genes was observed (Table 4.3, Figure 4.13, Figure 4.15 and Figure 4.21). The only exceptions were Syt10, for which no specific labeling could be detected, and Syt-6, which was only expressed at very low levels. Interestingly, within the granular cell layer individual cells showed a very strong labeling for Syt-1, potentially representing Golgi cells (GC). Syt-7 was most prominently expressed in the Purkinje cells (PC), however also Syt-2 and Syt-4 transcripts were clearly labeled in these cells. The molecular layer of the cerebellum (ML) is mainly devoid of Synaptotagmin transcripts, only hybridization with probes against Syt-2, Syt-6 and Syt-11 resulted in a weak labeling. In the
deep cerebellar nuclei, expression of all Synaptotagmin genes, except Syt-9, was observed.

4.2.2.9 Glial cells

Based on the ISH signals, in some cases, expression patterns of Synaptotagmins could be distinguished from neuronal expression already on a macroscopic level. Whereas no labeling was observed for neuron-specific genes in areas of high axonal density like the white matter regions or layer I of the cortex, hybridization of a probe to mRNA present in glial cells resulted in a strong signal in these regions. Syt-11 was the only Synaptotagmin isoform for which a marked ISH signal in putative glial cells was observed, e.g. a strong labeling was detected in the capsule, the corpus callosum and the fimbria of the hippocampus (Figure 4.15 and Figure 4.20).

4.2.3 Expression of RIM-BP genes

- identification of a new RIM-BP family member

RIM-binding proteins (RIM-BPs) were identified as binding partners of the pre-synaptic active zone proteins RIMs as well as for voltage gated Ca\(^{2+}\)-channels (Hibino et al. 2002, Wang et al. 2000). They were suggested to form a functional link between the synaptic-vesicle fusion apparatus and Ca\(^{2+}\)-channels. However, so far nothing is known about RIM-BP expression, regional distribution or genomic organization.

4.2.3.1 Identification of a third member of the RIM-BP gene family

While designing primers and probes for the RIM-BP genes, we identified a novel RIM-BP-related gene in addition to the previously described RIM-BP1 and RIM-BP2 genes (Hibino et al. 2002, Wang et al. 2000), referred to as RIM-BP3. This gene was related to existing ESTs and its existence was verified by cloning the full length sequence of this novel gene from mouse testes cDNA (Figure 4.23). The RIM-BP3 gene expresses a single transcript with a domain organization similar to RIM-BP1 and RIM-BP2 (Figure 4.22A). The primary structure of human and mouse RIM-BP3 consists of 1639 and 1606 amino acid residues, respectively, with a calculated molecular mass of around 180 kDa. The RIM-BP3 N-terminus exhibits only a low degree of homology with the N-terminal region of RIM-BP1. The amino acid composition of this region is characterized by
its high content of arginine, glutamic acid, aspartic acid, and serine (Figure 4.22B). Furthermore, sequence analysis revealed a mixed charge cluster within the first 120 AA and a proline rich region (Figure 4.22B). All RIM-BP proteins contain a central SH3-domain and a cluster of Fibronectin III (FNIII) repeats. However, whereas in RIM-BP1 and RIM-BP2 three FNIII repeats are found, only the second and third FNIII repeats are present in RIM-BP3. In their C-terminus all RIM-BP proteins are composed of two SH3-domains followed by sequences that diverge in length and composition between the three isoforms. Within the RIM-BP family RIM-BP3 is most closely related to RIM-BP1, both in size and in amino acid composition. Even though the overall identity for the full-length proteins is only about 21%, the SH3- and FNIII-domains exhibit a significant conservation with identities ranging from 41% to 75% (Figure 4.22C, Table 4.4).

Figure 4.22: Structure of the RIM-BP protein family. (A) Diagram of the domain composition of RIM-BPs. RIM-BPs share a similar domain organization with a SH3-domain and a cluster of Fibronectin type III (FNIII) repeats in the center of the protein and a doublet of SH3-domain in the C-terminus. Asterisks mark the three sites of alternative splicing in RIM-BP1 and -2. (B) The RIM-BP3 N-terminus contains an arginine-rich (AA 30-137), serine-rich (AA 214-330) and proline-rich (AA 312-366) region. (C) The RIM-BP SH3- and FNIII-domains are highly homologous, as shown here for SH3-1 and FNIII-3. Filled black boxes mark amino acids identical in all three human RIM-BP proteins and grey boxes indicate conserved residues.
Table 4.4: Identity and similarity of RIM-BP proteins

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<td>48% / 61%</td>
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<td>21% / 29%</td>
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Amino acid identities and similarities of protein sequences were determined the sequence manipulation suite (accessible at http://www.ualberta.ca/~stothard/javascript/ident_sim.html) after their pair-wise alignment using ClustalW (Gonnet protein weight matrix and settings of a gap open penalty of 10, a gap extension penalty of 0.05, and no gap separation penalty). Amino acids were counted as similar within the following groups (1) GAVLI, (2) FYW, (3) CM, (4) ST, (5) KRH, (6) DENQ, and (7) P.

4.2.3.2 Expression of the RIM-BP genes

Quantitative realtime PCR analyses of mouse tissues was performed with primers specific to RIM-BP1, -2 and -3 to elucidate their expression as compared to Actin, that was used as normalization control. RIM-BP1 and -2 were synthesized at high levels exclusively in brain of all tissues analyzed. RIM-BP3 mRNA in contrast was detected ubiquitously, with the highest level of expression in testes (Figure 4.23 A, B). To examine the regional expression of RIM-BPs within the brain, cDNA from different brain regions was prepared and RIM-BP mRNA levels were measured by RT-PCR. While RIM-BP1 exhibits a strong ubiquitous expression throughout the brain, high levels of RIM-BP2 mRNA can be detected in all brain regions with exception of the cerebellum. RIM-BP3 showed a low uniform expression throughout all brain regions (Figure 4.23C). During early brain development, starting at embryonic day 12.5 till birth (E12.5 - P0), all RIM-BP genes were expressed at an equally low level. However, whereas RIM-BP1 and -2 exhibited the strong increase in expression between P0 and P15 typically observed for synaptic proteins, RIM-BP3 expression levels remained unchanged (Figure 4.23D).
Figure 4.23: Expression of RIM-BP genes

(A) Amplification of full-length RIM-BP3 from mouse testes and p0 cortex cDNA. Control experiments were performed with template RNA that had not been reverse transcribed to rule out genomic contamination. (B, C, D) Quantitative real-time PCR expression profiles of mouse cDNA from different tissues (B), brain regions (C) and whole brain of different developmental stages (D). The expression level is depicted as the average of three independent reactions normalized to Actin, the error bar indicates the standard error of the mean (SEM).

To further investigate the neuronal distribution of RIM-BPs, radioactive in situ hybridizations on brain sections from adult and newborn rats was performed (Figure 4.24). Two oligonucleotide probes were used for each RIM-BP isoform to ensure that identical labeling patterns were obtained (data not shown). The labeling was abolished when excess unlabelled oligonucleotide probe was added to the hybridization mix (Figure 4.24 C, F). Due to the low abundance of RIM-BP3 mRNA in the adult rat brain (Figure 4.23 A to D) Even though four independent ISH probes against RIM-BP3 were tested, no specific signal could be detected (data not shown). Autoradiographs of hybridized rat brain sections revealed differential but overlapping expression patterns of RIM-BP1 and -2 mRNAs. Regions rich in glial cells (e.g. white matter of cerebral cortex and cerebellum) were unlabelled, indicating a neuron-specific expression of these two RIM-BP isoforms (Figure 4.24 A, D). RIM-BP1 mRNA was present throughout the brain with highest levels in the cerebellum, cortical layers II/III and V/VI, hippocampus and olfactory bulb. Striatum, thalamus and the pontine nuclei also show a strong RIM-BP1 expression (Figure 4.24A). RIM-BP2 mRNA was highly concentrated in the telencephalon, consisting of hippocampus, olfactory bulb,
and cortex, while it is expressed at lower levels in all other brain regions. Consistent with the RT-PCR data, it was barely detectable in the adult cerebellum (Figure 4.24D). The regional distribution of both RIM-BP1 and -2 detected in the adult brain already emerges at the day of birth (p0). RIM-BP1 could be detected ubiquitously in the p0 brain, with less abundance in white mater of cortex and brain stem (Figure 4.24B), while RIM-BP2 shows a more distinct expression pattern with a strong signal in the telencephalon (Figure 4.24E). However, whereas RIM-BP2 was barely detectable in the midbrain and brain stem at p28, expression levels were low in these areas at p0.

Figure 4.24: Localization of RIM-BP expression in rat brain.
(A, B, D, E) In situ hybridizations were performed on horizontal and sagittal sections of adult (p28) and new born (p0) rat brain using 35S labeled oligonucleotide probes. (A, D) Autoradiograph pictures of horizontal and sagittal sections of p28 rat brain show distinct expression patterns of RIM-BP1 (A) and RIM-BP2 (D) expression. (B, D) Film images of sagittal sections showing the distributions of RIM-BP1 (B) and RIM-BP2 (E) at p0. (C, F) Control experiments with 1000-fold excess of unlabeled oligonucleotide did not result in any labeling. BS: brain stem; TH: thalamus; CX: cortex; HC: hippocampus; CB: cerebellum; OB: olfactory bulb; STR: striatum; Pn: pontine nuclei.
4.2.3.3 Structure of the RIM-BP genes

The three RIM-BP genes are dispersed in the human and mouse genome. However, the genes are highly conserved between human and mouse, with a very similar arrangement of exons and introns, whereby the mouse genes are more condensed (Figure 4.25). The properties of the human and mouse RIM-BP genes are summarized in Table 4.5, the structures of the genes are depicted schematically in Figure 4.25, and the sizes of the exons and the sequences of the exon-intron junctions are presented in G7Table 4.6 – Table 4.9.

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**Mouse RIM-BP3**

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Figure 4.25: Structures of the human and mouse RIM-BP genes.

Diagrams depict the positions of the exons and introns in the RIM-BP genes (G7Table 4.6 – Table 4.9). Exons are identified by numbers; the first translated exon is marked by an arrow in RIM-BP2, asterisks mark exons subject to alternative splicing. The rulers above each gene diagram show the positions in the USCD database genome sequences.
Even though the RIM-BP1 gene codes for a large protein of 1907aa and 1895aa for the human and mouse protein, the human and mouse RIM-BP1 gene loci are relatively small with 27.5 and 24.5 kb, while the human and mouse RIM-BP2 genes measure 316.6 and 320.1 kb. This results in an inverse correlation between the size of the gene loci and the size of the corresponding protein, as the RIM-BP2 gene, encoding for the smaller RIM-BP2 protein with 1076aa (human) and 1079aa (mouse) is more than ten times the size of the RIM-BP1 gene. In contrast to the RIM-BP1 gene the human and mouse RIM-BP2 genes contain several non-coding exons in its 5’-UTR. The RIM-BP2 protein is mainly composed of a short N-terminal RIM-BP1 homology region and the SH3- and FNIII-domains that characterize the RIM-BP protein family. The homology domains are connected to each other by short linker sequences that diverge significantly between the three RIM-BPs. Therefore, positions of exon-intron junctions are only conserved within the functional domains between the RIM-BP1 and -2 genes. The RIM-BP3 gene, even though it encodes for a large protein, is contained within one exon of ~ 5,5 kb. In the human, in contrast to all other genomes analyzed, three copies of the RIM-BP3 gene can be found on chromosome 22 within 1.4 mb (Table 4.5).

**Table 4.5: Chromosomal locations and sizes of RIM-BP genes and proteins**

<table>
<thead>
<tr>
<th>Gene</th>
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The sequence homology between these three duplicates is very high, at 99% for the overall sequences, whereas the phylogenetic distance between the second and third RIM-BP3 (LOC440804 and LOC150221, respectively) is even lower than the distance of these both to RIM-BP3.1 (KIAA1666). This indicates that they did not replicate at once, but a second duplication event occurred to generate the third RIM-BP3.3 gene.

4.2.3.4 Alternative splicing of RIM-BPs

Analysis of EST and cDNA sequences in public databases revealed that the RIM-BP1 and RIM-BP2 transcripts each contain three sites of alternative splicing (Table 4.10; marked by asterisks in Figure 4.22A), that are conserved in human and mouse: The first in the N-terminal region, the second between the FNIII domain and the C-terminal SH3-domains and the third at the C-terminus.

Splicing at the N-terminal first splice site results in the inclusion or exclusion of exon 4 in the RIM-BP1 transcript, whereas in RIM-BP2 the alternatively spliced sequence is encoded by two very short exons (6 and 7) that are in frame and therefore can be independently spliced (G7Table 4.6 and Table 4.7, Figure 4.25). Interestingly, the second splice site is located between the FNIII-domains and the C-terminal SH3-domains. In RIM-BP1 the alternatively spliced cassette exon 22 is very large (840 bp) and comprises the majority of the linker region between the functional domains. Furthermore, this sequence contains one negative charge cluster consisting of a string of 11 consecutive glutamic acid residues and is rich in proline, serine, arginine and glycine. In the RIM-BP2 gene alternative splicing at the second splice sites results in the exclusion of exon 15 and the usage of alternative splice donor and acceptor sites in exon 14 and 16, respectively, leading to the deletion or insertion of two short 18- and 8-residue sequences (Table 4.8 and Table 4.9, Figure 4.25). Therefore, the second splice site impacts the distance between the FNIII- and SH3-domains in the RIM-BP2 gene as well. At the third splice site inclusion or exclusion of exon 31 of RIM-BP1 and intron retention between exon 22 and 23 in RIM-BP2 leads to the generation of two proteins with diverging C-terminal sequences.

No evidence for alternative splicing of the C. elegans RIM-BP transcript was detected. The Drosophila RIM-BP appears to be alternatively spliced at the N-terminus as ESTs encompassing three different transcript variants can be found in the databases.
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Data are based on the analysis of human genome sequences in the NCBI, Ensembl and USCD databases. Nucleotide numbers correspond to those of the assembled USCD genome.

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Data are based on the analysis of human genome sequences in the NCBI, Ensembl and USCD databases. Nucleotide numbers correspond to those of the assembled USCD genome.

*Exons that are subject to alternative splicing.
Table 4.8: Exon-intron structure of the human RIM-BP2 gene

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Data are based on the analysis of human genome sequences in the NCBI, Ensembl and USCD databases. Nucleotide numbers correspond to those of the assembled USCD genome. Exons with “a” and “b” forms contain alternative splice donor or acceptor sequences and “c” marks an intron retention.

*Exons that are subject to alternative splicing.
Table 4.9: Exon-intron structure of the mouse RIM-BP2 gene

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Data are based on the analysis of mouse genome sequences in the NCBI, Ensembl and USCD databases. Nucleotide numbers correspond to those of the assembled USCD genome. Exons with “a” and “b” forms contain alternative splice donor or acceptor sequences and “c” marks an intron retention.

*Exons that are subject to alternative splicing.
Table 4.10: Supporting transcripts for alternatively spliced exons in RIM-BP1 and -BP2

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4.2.3.5 Evolution of RIM-BP genes

In the ENSEMBL Databank various members of the RIM-BP gene family were identified in the whole set of genomes presently available. Genomic alignments were used to select the species where the sequenced genome covered all potential RIM-BP genes (Table 4.11).

Figure 4.26: Conservation of RIM-BP domain structure and sequence during evolution.

(A) Domain structure of vertebrate and invertebrate RIM-BP proteins. (B) High sequence homology within the conserved domains exemplified by a ClustalW alignment of the second SH3 domain of vertebrate and invertebrate RIM-BP sequences. Darker boxes represent higher degrees of homology.

A single RIM-BP gene was identified in C. elegans as the product of the tag-168 gene and in Drosophila as the product of the CG31302 gene (Figure 4.26, Figure 4.27). Furthermore, the genomes of Anopheles gambiae, aedes aegypti and ciona intestinalis contain one single RIM-BP gene, while all vertebrate genomes analyzed contain at least two and up to five RIM-BP genes. The transcripts were aligned with T-Coffee [http://tcoffee.vital-it.ch/cgi-
bin/Tcoffee/tcoffee_cgi/index.cgi] (Notredame et al. 2000), using the longest predicted transcript variants in case of possible alternative gene assembly.

The RIM-BP genes can be classified as members of the RIM-BP gene family based on their domain composition of three SH3- and three contiguous FNIII-domains and the structural organization of these domains that is identical in vertebrate and invertebrate RIM-BPs (Figure 4.26A). These signature domains are highly conserved in all species analyzed, exemplified by the alignment of the second SH3 domain of various vertebrate and invertebrate RIM-BP sequences (Figure 4.26B). In contrast to these highly homologous regions, the linker sequences connecting these domains exhibit almost no similarity between invertebrates and vertebrates or even between various invertebrates. The C. elegans and Drosophila RIM-BP genes are complex and rather large genes (~17.5 kb with 20 exons for C. elegans, and ~16.8 kb with 14 exons for Drosophila, Figure 4.27). However, the gene structures differ greatly between the different invertebrates and the vertebrates. In the human and mouse genome, introns in the two vertebrate multi-exon RIM-BP genes are located at identical positions and the exons have the same length. The C. elegans and Drosophila gene structures are neither similar to the vertebrate RIM-BP1 nor the RIM-BP2 genes (Figure 4.27).

The single exon RIM-BP3 gene can only be found in therian mammals. In the prototherian platypus Ornithorhynchus anatinus, only one RIM-BP1 and -2 homolog was discovered. RIM-BP3 is present in one copy in the genomes of the marsupial monodelphis domestica and the eutherians bos taurus, canis familiaris, mus musculus, rattus norvegicus, macaca mulatta, and pan troglodytes. Interestingly, three copies of the RIM-BP3 gene are located in close proximity on chromosome 22 in homo sapiens. All three transcripts were identified by the SEGE intronless Genes in Eukaryotes database as functional intronless genes [http://sege.ntu.edu.sg/wester/intronless/search.htm] (Sakharkar & Kangueane 2004). In some of the lower vertebrates that lack RIM-BP3, the alignments also resulted in three or even four RIM-BP genes. The phylogenetic analysis with PHYML, a tool to estimate maximum likelihood phylogenies of DNA and protein sequences [http://atgc.lirmm.fr/phylm] (Guindon & Gascuel 2003, Guindon et al. 2005), confirmed in these cases that RIM-BP1 or both RIM-BP1 and -2 had been replicated (Figure 4.28). In the only bird genome available, the genome of
the chick (*Gallus gallus*), two RIM-BP1 genes were found. A second copy of both RIM-BP1 and -2 is present in the genomes of all *Teleostei* of the suborder *Acanthopterygii* that were available at the time (Table 4.11). However, in the zebra fish, *Danio rerio*, representing the suborder *Ostariophysi*, only one RIM-BP gene of each kind is present in the genome. The resulting phylogenetic tree (Figure 4.28) shows the great distances in homology between nematodes (*C. elegans*), arthropods (*Anopheles, Drosophila*) and Chordates (*Ciona*). The analysis also revealed that the vertebrate RIM-BP2 genes seem to be most closely related to the other phyla, with RIM-BP1 and RIM-BP3 diverging thereafter.
Figure 4.28: Phylogenetic tree of RIM-BPs from various organisms including nematodes, arthropods, chordates and higher vertebrates

Phylogenetic distances between the organisms were calculated with PHYML [http://atgc.lirmm.fr/phyml]. Numbers at the branches are confidence values based on the bootstrap method. B = 100 bootstrap replications (Felsenstein 1985)
Table 4.11: ENSEMBL gene annotations of the RIM-BP family genes

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PT: Prototheria; *The O. anatinus gene is encompassed in two independent contigs in ENSEMBL, each part represented by one annotation.