5.3 Conclusions and Outlook

The data presented in this thesis indicates that α2δ subunits of VGCCs interact with AMPARs and alter their function. It was conclusively and reproducibly shown that the coexpression of either α2δ-1 or α2δ-3 decreases desensitizing steady-state currents of GluA1(Q)flip in X. laevis oocytes. Not only was this repeatedly demonstrated in the experiments discussed in Section 5.1, but when normalized data of all recordings done in the scope of this thesis were combined, there was a highly significant reduction of glutamate-evoked current amplitudes of 33% by α2δ-1 and 40% by α2δ-3 (Section 4.2.1.9, Figure 4.11); concurrently, amplitudes of non-desensitizing currents were unaltered. Combined with the differential effects on the flip and flop splice variants of GluA1 and the data for the non-desensitizing L483Y mutation, which was not affected by α2δ coexpression, this strongly indicates a modulation of the desensitization behavior of GluA1 by the VGCC auxiliary subunits. Strikingly, the extent of this modulation was less pronounced for GluA2 and GluA3, and completely absent for GluA4. A phylogenetic analysis of the four AMPARs over their whole amino acid sequence and the sequences of the extracellular domains provides a possible explanation for this (Figure 5.2). The phylogenetic distances of the full-length sequence (A), the ATD (B), the LBD consisting of S1 and S2 (C), and the isolated S1 domain (D) do not show a pattern that could explain the differential modulation. But when the sequence of the isolated S2 domain is analyzed (E), it can be seen that the sequence of GluA4(Q)flip is the farthest removed from that of GluA1(Q)flip, while those of GluA2 and GluA3 are in between. This corresponds with the differential modulation of the flip and flop isoforms of GluA1, since the flip/flop splicing cassette is located in the S2 domain, and residues in S2 are determinative for desensitization of the receptor. It is therefore concluded that the α2δ subunits, which are fully extracellular proteins anchored to the membrane by GPI, interact with the S2 domain of GluA1, possibly accelerating the time course of desensitization.

However, this could not be reproduced when GluA1(Q)flip was coexpressed with the α2δ subunits in HEK cells. Although α2δ-1 did alter the time course of desensitization and the open probability of the receptor, these changes would translate to larger steady-state currents in oocytes. Resembling the modulation of AMPARs by type I TARPs (Tomita, Adesnik, et al., 2005; Cho et al., 2007), receptor open probability was increased by α2δ-1 and the time course of desensitization was slowed down. There are two possible explanations for this discrepancy. One option is the requirement for a certain
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Figure 5.2: Phylogenetic trees resulting from multiple sequence alignment of the four AMPARs and their extracellular domains. Phylogenetic relationships are shown for the full-length receptors (A), the ATD (B), the combined LBD consisting of S1 and S2 (C), the S1 domain (D), and the S2 domain (E). The sequences of the S1 and S2 domain included the respective linker domains.

Stoichiometry between the AMPAR and the α2δ subunit. In oocytes, expression levels rise rather slowly over the course of several days, and it was shown in the time course experiments that the extent of α2δ modulation was in fact dependent on the recording day. However, due to the large inter-batch variability in expression levels and rates, no general conclusion can be reached from these data. HEK cells, on the other hand, express transiently transfected proteins quite rapidly, and expression levels change over the course of hours. Ideally, patch clamp recordings would have been carried out over a time range beginning several hours after transfection. However, recordings of outside-out patches from double-transfected cells come with several constraints prohibiting such experiments: The cells cannot be transfected in glass-bottom dishes (see Section 3.2.2.3), but these are required for the identification of transfected cells by fluorescence; pulling of outside-out patches requires cells that firmly adhere to the dish, which takes several hours after splitting. Therefore, a potential stoichiometric “sweet spot” could have been missed due to the late time of recording. However, in this case a complete lack of modulation would have been expected, and not the contrary effects on open probability and desensitization that were observed.

In the light of these findings, the second option becomes more likely: The interaction between GluA1 and the α2δ subunits requires at least one additional, unknown protein that is endogenously present in X. laevis oocytes, but not in HEK cells. While this may seem unlikely at first, since amphibian oocytes intuitively are a more artificial expression system than a human cell line, they do in fact express several proteins endogenously that are similar to mammalian neuronal proteins and can interact with those. For example,
it is known that *X. laevis* oocytes express homologs of all known mammalian iGluRs on the mRNA level (Schmidt, Klein, et al., 2009), and a GluN2 subunit, *XenNR2*, on the protein level, which can form functional ion channels with exogenous GluN1 subunits (Schmidt and Hollmann, 2008). Furthermore, they possess several endogenous VGCCs (Dascal et al., 1986; Dascal, 1987; Leonard, Nargeot, et al., 1987; Lacerda, Perez-Reyes, et al., 1994; Terhag, Cavara, et al., 2010). If it is assumed that they do not only possess these ion channels, but also auxiliary subunits and associated proteins, this opens up a wide spectrum of possible adapter proteins required for the interaction between $\alpha_2\delta$ subunits and GluA1. This idea is corroborated by the experiments with GluA1(Q)flip labeled with EGFP at the CTD, in which no modulation of receptor function was observable. The CTD is known to mediate interactions with several intracellular proteins (Leonard, Davare, et al., 1998; Dong et al., 1997; Xia et al., 1999) and is therefore a conceivable candidate for interaction with the putative adapter protein. Obviously, the $\gamma$ subunits, which have been shown to interact with AMPARs as well as with VGCCs, could theoretically provide this adapter function, but so far no endogenous expression of these subunits has been shown in oocytes.

Of course the possibility that the results presented and discussed in this thesis are mere artifacts of the *X. laevis* oocyte expression system cannot be ruled out. However, the study by Kang, Chen, et al. (2006), which was the basis for this work, definitely showed the existence of AMPAR-VGCC complexes *in vivo*. And although the authors show no co-immunoprecipitations made with antibodies directed against $\alpha_2\delta$, they did show the presence of these subunits in the PSD, which allows the conclusion that AMPARs and $\alpha_2\delta$ subunits are in close proximity to each other *in vivo*.

In the past years, several studies aimed to identify possible auxiliary subunits by proteomic approaches. This led to the identification of the cornichons homologs (Schwenk, Harmel, Zolles, et al., 2009), CKAMP44 (von Engelhardt et al., 2010), and GSG1L (Shanks, Savas, et al., 2012) as proteins that definitely interact with AMPARs, and a large number of other proteins that are part of AMPAR complexes (Schwenk, Harmel, Brechet, et al., 2012). Although none of these studies, which were all based on co-immunoprecipitation, showed an $\alpha_2\delta$ subunit as a constituent of an AMPAR complex, this does not exclude the possibility that $\alpha_2\delta$ subunits interact with AMPARs *in vivo*. While co-immunoprecipitation is an invaluable tool for the discovery of protein-protein interactions, it also relies on the stability of these interactions for the detection of proteins bound to the receptor indirectly. In fact, the method of sample preparation and the choice of the purification method have great impact on results of these experiments (Kang, Nuriya, et al., 2012). If the interaction between GluA1 and an $\alpha_2\delta$ subunit relied on additional adapter proteins as suggested above, it is well conceivable that this interaction would have been missed in the proteomic approaches.

According to Yan and Tomita (2012), for a protein to be defined as an auxiliary subunit of glutamate receptors, it needs to meet four requirements: It has to be a non-pore-forming subunit, it has to exhibit direct and stable interactions with a pore-forming subunit, it needs to modulate the channel properties and/or trafficking in heterologous expression systems, and it must be necessary *in vivo*. The first requirement is a given for $\alpha_2\delta$ subunits, and this thesis provides proof for the third. The proteomics studies cited
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in the preceding paragraph suggest that the second requirement is not met. While this could mean that αδ subunits may not qualify as true auxiliary subunits, they remain potential interactors, albeit the interaction being of weak or transient nature.

The fourth criterion, physiological necessity, remains to be elucidated. Although an αδ-1 knockout mouse is described in the literature (Fuller-Bicer et al., 2009), the authors unfortunately only investigated the influence of this knockout on cardiac properties, but the animals were viable and the authors did not mention any neurological phenotypes. On the other hand, it is not necessarily expected that the modulations shown in this thesis translate to a visible phenotype comparable to that of the stargazer mouse: TARPs strongly increase surface expression of AMPARs and boost their glutamate-evoked current responses 10 to 400-fold, depending on the TARP and the AMPAR subunit and splice variant (Kott et al., 2007). In contrast to that, the modulation by the αδ subunits described here is rather modest, decreasing currents by 30% to 50%.

Interestingly, several neuropathies are linked to both AMPARs and αδ subunits. AMPARs are hypothesized to be involved in schizophrenia directly (Hammond et al., 2011; Tucholski et al., 2013) as well as indirectly (Seebohm et al., 2014), and there is evidence for an upregulation of Cornichons in schizophrenia patients (Drummond et al., 2012). The αδ subunits were also linked to schizophrenia in a genetic study (Purcell et al., 2014). Furthermore, αδ-1 is the molecular targets of gabapentinoid drugs like gabapentin and pregabalin (Gee et al., 1996), which are used in the treatment of epilepsy and neuropathic pain (for review see Taylor et al., 2007). Similarly, AMPARs have recently come into focus as molecular targets for anticonvulsants like perampanel (Rogawski and Hanada, 2013).

Since the cloning of the first ionotropic glutamate receptor 27 years ago, our understanding of iGluR function has come a long way. Jump-started by the discovery of the TARPs at the turn of the millennium, an ever-growing network of auxiliary and interacting proteins has been unraveled, fueled by the establishment of high-throughput techniques and constant lowering of detection thresholds.

In contrast to many of the recent studies that employed shotgun approaches, this thesis constitutes a bottom-up approach in the identification of novel AMPAR interacting proteins. Its aim was not to prove the physiological relevance of these putative interactors, but rather to serve as a stepping stone for future research. While it was established that αδ and αδ-3 modulate AMPAR function in oocytes, the exact nature of this modulation remains elusive. Further experiments, for example domain-transplantation experiments or alanine scans, are required to pinpoint the sites of interaction. For a more physiological approach, siRNA-mediated knockdown of αδ subunits in primary neurons could be attempted, or it could be tried to obtain tissue or cells from αδ knockout animals, to perform patch clamping experiments under more physiological conditions. Furthermore, the effects of gabapentinoid drugs on the modulation of AMPARs by αδ-1 could be studied, potentially leading to a better understanding of the mechanisms behind epilepsy and its treatment.