Physiological characterization of purinergic signaling in single spermatogonia *in vitro* and *in situ*.

Von der Fakultät für Mathematik, Informatik und Naturwissenschaften der RWTH Aachen University zur Erlangung des akademischen Grades eines Doktors der Naturwissenschaften genehmigte Dissertation

vorgelegt von

Master of Science Biology

David Fleck

aus Aachen

Berichter: Universitätsprofessor Dr. rer. nat. Marc Spehr
Universitätsprofessor Dr. med. Günther Schmalzing

Tag der mündlichen Prüfung: 14.03.2016

Diese Dissertation ist auf den Internetseiten der Universitätsbibliothek online verfügbar.
# Contents

1 Introduction 1

1.1 Physiology of the Testis 1

1.1.1 Structure and Function of the Testis 1

1.2 Spermatogenesis 3

1.2.1 Cycle of the Seminiferous Epithelium 3

1.2.2 Spermatogonia 4

1.2.3 Germ Cell Maturation is Guided by Sertoli Cells 6

1.3 Purinergic Signaling 9

1.3.1 Purinergic signaling mechanisms 9

1.3.2 P2X Receptors 10

1.3.3 Purinergic Signaling in the Testis 12

1.4 Tissue Clearing Techniques 13

1.5 Aims 15

2 Materials and Methods 17

2.1 Material 17

2.1.1 Solutions 17

2.1.2 Nucleic Acids 20

2.1.2.1 Primers 20

2.1.2.2 Anti-sense RNA 21

2.1.3 Chemicals 21

2.1.4 Equipment 25

2.1.5 Mouse strains 26

2.1.6 Setups 26

2.1.6.1 Leica DMI4000 B 27

2.1.6.2 Leica DM6000 FS 27

2.1.6.3 Leica Confocal SP5 28

2.1.6.4 Leica Multiphoton SP8 28

2.1.7 Software 29
2.2 Methods .......................................................... 29
  2.2.1 Cell Culture .................................................... 29
    2.2.1.1 Spermatogonia Cell Culture .......................... 29
    2.2.1.2 RNA-Interference (RNAi) Transfection of Cultured Sper-
    matogonia ......................................................... 30
  2.2.2 Molecular Methods .......................................... 30
    2.2.2.1 RNA Isolation of Cultured Germ Cells and Tissue Prepa-
    rations (Testis, Brain, Spinal Cord) ....................... 30
    2.2.2.2 RT-PCR of Cultured Germ Cell RNA and Tissue RNA
    Extracts (testis, brain, spinal cord) ...................... 31
    2.2.2.3 Gel Electrophoresis ................................. 32
    2.2.2.4 Quantitative-PCR .................................... 32
  2.2.3 Preparation of Vibratome Slices from Seminiferous Tubules . . 33
  2.2.4 Electrophysiology .......................................... 33
    2.2.4.1 General Description of Whole-cell Patch-clamp Experi-
    ments ........................................................... 33
    2.2.4.2 Methodical Description of Whole-cell Patch-clamp Ex-
    periments ....................................................... 34
    2.2.4.3 Electrophysiological Recordings of Cultured Spermato-
    gonia ............................................................ 35
    2.2.4.4 Electrophysiological Recordings of Acute Tissue Slices
    from Seminiferous Tubules ................................. 35
  2.2.5 Immunohistology ........................................... 35
    2.2.5.1 Cryosections and Immunostainings of Testis Tissue ... 35
    2.2.5.2 Immunoblotting ...................................... 36
  2.2.6 Data Analysis ............................................... 36
  2.2.7 CLARITY .................................................... 37
    2.2.7.1 Whole Animal Perfusion Fixation for Rodents ........ 37
    2.2.7.2 Hydrogel Tissue Embedding .......................... 37
    2.2.7.3 Electrophoretic Tissue Clearing (ETC) .............. 38
    2.2.7.4 Imaging of Cleared Samples .......................... 38
  2.2.8 Scanning Electron Microscopy ............................. 39
3 Results

3.1 Ion Channels in Spermatogonia

3.1.1 Electrophysiological Characterization of ATP-induced Responses in Prepubescent Spermatogonia

3.1.2 Varying the Membrane Potential Reveals two Different ATP-induced Responses.

3.1.3 Molecular Analysis of P2X Receptors in the Testicular Tissue

3.1.4 Identifying the channels activated by low ATP concentrations

3.1.5 High ATP Concentrations Activate Additional Low Sensitivity ATP Receptors

3.1.6 Spermatogonia and Sertoli Cells Show Typical ATP-induced Currents

3.2 Excursus 1: Deletion of GAR22β gene impairs spermatogenesis and spermatozoa motility

3.3 Excursus 2: CLARITY for Rapid Clearing and Imaging of Intact Anatomical Structures

4 Discussion

4.1 Electrophysiological Experiments with Extracellular ATP in Prepubescent Spermatogonia

4.1.1 Targeting Spermatogonia

4.1.2 Identifying ATP-sensitive Channels in Spermatogonia

4.1.3 Selective Pharmacological Ion Channel Inhibitors and Modulators

4.1.4 Calcium-activated Potassium Channels Mediate the Delayed Current.

4.1.5 High ATP Concentrations Activate Additional Low Sensitivity ATP-Receptors

4.1.6 RNAi Knockdown of P2X Receptors in the Testicular Tissue

4.1.6.1 RNAi Knockdown of P2X2

4.1.6.2 RNAi Knockdown of P2X4

4.1.6.3 RNAi Knockdown of P2X7

4.1.7 Spermatogonia and Sertoli Cells Show Typical ATP-induced Currents

4.2 Excursus 1: Deletion of GAR22β Gene Impairs Spermatogenesis and Spermatozoa Motility
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.3 Excursus 2: CLARITY for Rapid Clearing and Imaging of Intact Anatomical Structures</td>
<td>93</td>
</tr>
<tr>
<td>5 Summary</td>
<td>95</td>
</tr>
<tr>
<td>References</td>
<td>96</td>
</tr>
</tbody>
</table>
1.1 Physiology of the Testis

1.1.1 Structure and Function of the Testis

Generating spermatozoa is crucial for the survival of a species. Spermatozoa are produced within the testes in a physiological process called spermatogenesis. The testes are encapsulated by a protective layer of dense connective tissue and smooth muscle fibers: the tunica albuginea. Each testis contains numerous seminiferous tubules and, between the tubules, interstitial tissue (figure 1.1; Fawcett et al., 1973). There are some variations across species. This thesis focuses on mouse spermatogenesis. In mice there are 10-15 tubules per testis with a total length of 2-3 m. Both ends of each tubule are connected to the rete testis (Clermont and Huckins, 1961; Nel-Themaat et al., 2009). Each seminiferous tubule is encapsulated by a layer of peritubular cells. These cells form the lamina propria, which builds the outer basement membrane of the seminiferous tubules (Tung, 1990; Mayerhofer, 2013).

Sertoli cells represent the only somatic cell type of the seminiferous epithelium (Russell et al., 1993). Sertoli cells span the entire epithelium and are thus in contact with both the basement membrane and the lumen of the seminiferous tubules. They form a tight junction barrier called blood-testis barrier (BTB), separating the basal and the adluminal compartment of the seminiferous tubule (Dym and Fawcett, 1970). Sertoli cells stay connected to germ cells of all developmental stages as nurse cells (Griswold and McLean, 2006).

According to their differentiation, germ cells represent the second type of cells within the seminiferous tubules. Premeiotically, these cells stay connected to each other af-
ter incomplete divisions via cytoplasmic bridges (Fawcett, 1959). Germ cells within the seminiferous epithelium are spermatogonia (diploid), spermatocytes (haploid) and spermatids (haploid). Spermatogonia are in contact with Sertoli and peritubular cells. Spermatocytes are in contact with Sertoli cells during their passage through the BTB. In the adluminal compartment, spermatocytes and spermatids are present. Mature, but immotile spermatozoa are shed from the epithelium and transported through the lumen of the seminiferous tubules (Russell et al., 1983). From there, spermatozoa are transported into the rete testis and into the epididymis. The epididymis is a single highly coiled duct that links the rete testis to the vas deferens. After several important maturation steps in the epididymis fertile spermatozoa are stored in the vas deferens until ejaculation (Robaire et al., 2006).

The interstitial tissue mainly consists of Leydig cells, monocytes, macrophages, T cells, natural killer cells, blood vessels, lymphatic vessels and some nerve fibers (Kerr et al., 2006). The Leydig cells are distributed in groups between the seminiferous tubules and the vasculature.

In addition to the production of fertile sperm the testes are also important for steroid generation. As part of the hypothalamic–pituitary–gonadal axis (HPG), the testes are regulated in a closed feedback loop with the other endocrine glands (O’Donnell et al., 2006). The hypothalamus dominantly controls the reproductive axis (Schlatt and Ehmcke, 2014). Gonadotropin-releasing hormone (GnRh) is secreted in the brain by the hypothalamus and acts on the pituitary endocrine gland that releases luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the vasculature. FSH is a mitogenic hormone that regulates Sertoli cell number around birth (Kerr et al., 2006). FSH stimulation of Sertoli cells regulates the proliferation of premeiotic germ cells throughout the seminiferous tubules in adult mice and therefore the efficiency of spermatogenesis (Schlatt and Ehmcke, 2014). Sertoli cells also rely on androgen stimulation to maintain spermatogenesis (De Gendt et al., 2004). Androgens are released in response to endocrine LH stimulation of Leydig cells (Steinberger, 1971; Saez, 1994; Carreau and Hess, 2010). Androgens also acts in a negative feedback loop on the hypothalamus. Additionally, androgens have other important functions throughout the male body and are responsible for secondary sexual characteristics (Jost et al., 1970).
Figure 1.1: Structure of the mouse testis and stages of the seminiferous cycle: Schematic drawing depicting the structure of the adult mouse testis. Right: the testis with parts of the tunica albuginea removed to visualize the seminiferous tubules. Additionally, the epididymis and the vas deferens are depicted. Left: Diagram illustrating the different cell types and stages within the seminiferous tubule during germ cell transition within the seminiferous cycle. Germ cells are marked in red, Sertoli cells are shown in beige. The blood testes barrier is depicted as red dashed line (seminiferous stages adapted from Russell et al. 1993).

1.2 Spermatogenesis

1.2.1 Cycle of the Seminiferous Epithelium

Spermatogenesis is a complex process. In general, undifferentiated germ cells, which reside at the basement membrane, develop into differentiated spermatozoa while moving towards the adluminal area of the seminiferous tubule. Eventually, mature spermatozoa are shed into the tubule’s lumen. The cellular developmental steps are not randomly distributed over the seminiferous tissue. The process can be divided in cellular associations or ‘epithelial stages’ of spermatogenesis which are timed precisely and appear highly organized (Leblond and Clermont, 1952). This organization is essential for continuous sperm production and depends on different factors, both paracrine (within the seminiferous tubules) and endocrine (by the HPG) (Hess and Franca, 2008). The division into stages is species-specific. In mice, seminiferous tubules undergo twelve consecutive
cyclical stages (I-XII; see figure 1.1; Leblond and Clermont, 1952; Oakberg, 1956). It takes 8.65 days to complete a cycle (Sirlin and Edwards, 1957; Hermo et al., 2010). Each germ cell runs through each of the twelve seminiferous stages 4.5 times. Thus, the development of one single spermatogonium to up to 4,096 spermatozoa takes about 39 days (Russell et al., 1993; Brinster and Zimmermann, 1994). However, it has been shown that under physiological conditions only approximately 25% of the theoretically generated germ cells develop to fertile spermatozoa (Kerr et al., 2006).

The highly organized seminiferous cycle is driven by precise programs in gene expression of both Sertoli and germ cells (Johnston et al., 2008). In germ cells, more than 2,300 genes are differentially expressed during maturation. The majority of these genes are expressed by premeiotic germ cells (Schultz et al., 2003). These genes have to be regulated precisely in the interplay with and between Sertoli cells to preserve the seminiferous cycle’s synchrony. It has been shown that germ cells have a predominant role in determining the duration of the seminiferous cycle (Russell and Brinster, 1996). One Sertoli cell is in contact with up to 50 germ cells at any given developmental stage (Weber et al., 1983; Hess and Franca, 2008). Sertoli cells are responsible for maintaining the organization of the stages during the seminiferous cycle (Ventelä et al., 2002). Moreover, extratubular factors influence spermatogenesis. Germ cell maturation, for instance, is controlled by the HPG. Sertoli cells alter their sensitivity to androgens and FSH during the seminiferous cycle to react stage specifically to external simulation (Tan et al., 2005).

1.2.2 Spermatogonia

Spermatogonia are the starting point of spermatogenesis. These cells allow lifelong sperm production after onset of spermatogenesis at puberty. Spermatogonia divide with an incomplete telophase, which leads to syncytia of spermatogonia connected via cytoplasmic bridges (Fawcett, 1959).

Spermatogonia are divided into multiple subpopulations. Historically, three populations were separated according to their heterochromatin shape (Oakberg, 1956; Chiarella et al., 2004). Spermatogonia consecutively develop from type A spermatogonia via intermediate (A_{int}) to type B spermatogonia. Type A spermatogonia can be further subdivided into undifferentiated and differentiating spermatogonia (Oakberg, 1971). The undifferentiated spermatogonia are then divided according to their syncytium size (A single (A_s), A paired (A_p) and A aligned (A_{al}(4-32))). A subpopulation of A_s and A_p spermatogonia serves as spermatogonial stem cells (SSC). Together with the A_{al} spermatogonia, SSCs develop independently of the seminiferous cycle. However, there is
an ongoing debate if $A_{al}$ spermatogonia could escape their fate of becoming developing spermatogonia by being fragmented again (Nakagawa et al., 2007, 2010; de Rooij and Griswold, 2012; Hogarth et al., 2013; Hara et al., 2014).

![Diagram](image)

**Figure 1.2: Stem cell renewal and transition of undifferentiated to differentiating spermatogonia:** Schematic illustration of stem cell transition and renewal after fragmentation (Nakagawa et al., 2007, 2010). Potential SSCs can replace actual SSCs in case of fragmentation and stem cell loss. The transition to A1 spermatogonia proceeds without mitotic division. The undifferentiated spermatogonial population consists of the $A_s$, $A_{pr}$ and $A_{al}$ spermatogonia (shown in light blue). A1 spermatogonia are the first members of the developing pool of spermatogonia (shown in dark blue) (modified from de Rooij and Griswold, 2012).

$A_{al}$ spermatogonia usually transform to A1 differentiating spermatogonia at step VIII of the seminiferous cycle (Chiarini-Garcia and Russell, 2001). In contrast to other developmental steps, this transition takes place without a cell division and includes the whole $A_{al}$ syncytium. These differentiating spermatogonia are divided into A1, A2, A3, A4, $A_{int}$ and B spermatogonia. These germ cell stage developments are synchronized to the seminiferous cycle. The cells divide after each developmental stage (de Rooij and Russell, 1997).

Type B spermatogonia turn into preleptotene spermatocytes. At this point, the whole syncytium detaches from the basement membrane of the seminiferous tubule (Russell, 1977, 1980; Smith and Braun, 2012). Preleptotene spermatocytes mark the beginning of meiosis. During their development, spermatocytes are transported by Sertoli cells to the adluminal compartment of the seminiferous tubules. After completion of meiosis I and II, the germ cells are called haploid spermatids. These rounded spermatids are
converted into elongated, mature and highly condensed spermatozoa in a process called spermiogenesis. Spermatogenesis finalizes with spermiation in which spermatozoa are released into the seminiferous tubules’ lumen.

1.2.3 Germ Cell Maturation is Guided by Sertoli Cells

The seminiferous tubule is a circular monolayer constructed by Sertoli cells. Each Sertoli cell can support up to 50 developing germ cells (Cheng and Mruk, 2010). Sertoli cells provide a separation of the testis into two functional compartments via the BTB. The BTB was first noted upon comparison of the peritubular and the luminal fluid of the seminiferous tubules. The luminal fluid differs significantly in respect of ionic and hormonal composition (Dym and Fawcett, 1970; Setchell, 1980). The BTB is a tight junction barrier separating the basal part of the seminiferous tubules from the adluminal part, where spermatocytes undergo meiosis and spermiogenesis takes place. It restricts the diffusion of molecules into the adluminal compartment and protects the germ cells against toxins and autoimmune responses by generating an immune-privileged compartment (Cheng and Mruk, 2012). The BTB remains unaffected after the removal of germ cells, showing that the presence of germ cells is of minor importance for BTB function (Kerr et al., 2006). Furthermore, adherens junctions supplement the contacting surfaces of adjacent Sertoli cells (Cheng and Mruk, 2002).

The position of individual germ cell stages is precisely synchronized with the seminiferous cycle (Oakberg, 1956). For instance, at stage VIII of the seminiferous cycle, spermatocytes translocate from the basal towards the adluminal compartment and cross the BTB while mature spermatids undergo spermiation. Both events require extensive restructuring at the interface of Sertoli-germ cells and at the BTB. Thus, on opposite sides of the Sertoli cell distinctive cellular events take place simultaneously. To parallelize different cellular events across the seminiferous epithelium, the BTB is part of a functional axis that persists locally in the seminiferous tubules. This functional axis continues in the adluminal compartment of the seminiferous tubules with the apical ectoplasmic specialization (ES). The apical ES is a testis-specific atypical anchoring junction type, connecting spermatogonia, spermatocytes and round spermatids with Sertoli cells (Cheng and Mruk, 2010). It allows Sertoli cells to move diverse developing germ cells within the seminiferous tubules to distinct positions. Therefore, a variety of specialized actin and microtubule (MT) networks span throughout the Sertoli cells. This network is connected to tight junction, desmosome-like and ES complexes between both Sertoli cells and germ cells (Mruk and Cheng, 2004; Cheng and Mruk, 2011). Germ cells remain
immotile throughout testicular maturation and fully rely on the Sertoli cells capability to translocate associated germ cell types. Cell Culture of Spermatogonia

Seminiferous tubules of adult mice have a larger diameter than those observed in prepubescent mice, and the adult germ cells are arranged around a lumen which allows the release of spermatozoa (see Fig. 1.3; A and B). In juvenile animals this lumen is not yet developed (see Fig. 1.3; C and D).

Seminiferous tubules from adult mice consist of peritubular cells, Sertoli cells and various germ cell stages. Immunohistochemical labeling of the protein 'deleted in azoospermia-like' (DAZL), a marker for spermatogonia and early spermatocytes (Ruggiu et al., 1997), shows that tubules from adult mice contain both numerous DAZL-positive spermatogonia at the basal membrane and many DAZL-negative germ cells in more apical areas, likely representing postmeiotic germ cell stages (figure 1.3; E and F). However, tubules from P7 mice contain only spermatogonia and Sertoli cells (Bellve, 1977; see Fig. 1.3; G and H).

Germ cells require a complex environment of endocrine and paracrine factors for their survival. Moreover, intimate contact to somatic cells – Sertoli cells – is a requirement for proper germ cell development. Therefore, a feeder layer of nursing cells is necessary to maintain vital germ cells under cell culture conditions. These requirements are met by co-cultured Sertoli cells. In culture, Sertoli cells grow as a confluent monolayer of large flat cells. Spermatogonia can grow on top of this feeder layer in close proximity to the Sertoli cells. Thereby, Sertoli cells provide necessary paracrine factors that supplement the culture medium (see Fig. 1.3; L; Veitinger et al., 2011).

To obtain a spermatogonial culture at the largest possible degree of purity, testicular cells from P7 mice can be isolated. Culturing these cells results in many small round cells lying on top of a flat Sertoli cell layer (figure 1.3; L) Veitinger et al. (2011). Immunocytochemical labeling of DAZL revealed that virtually all round cells were labeled and therefore are likely to represent spermatogonia (figure 1.3; I-K). Moreover, single as well as paired and aligned spermatogonia are found using this cell culture technique (Veitinger, 2009).
Figure 1.3: Different tissue composition of prepubescent versus adult seminiferous tubules. (A - D) Scanning electron microscopy (SEM) micrographs of freeze-fractured adult (A and B) and prepubescent (postnatal day (P) 7) (C and D) testes. Several seminiferous tubules have been fractured revealing their cellular composition. (B and D) are higher magnifications of the tubules marked in (A) and (C), respectively. At both ages, several cell layers are visible. However, in the prepubescent tubules, a lumen has not developed yet. (E-H) Testicular cryosections from adult (E and F) and P7 (G and H) mice immunostained for DAZL. As a marker for premeiotic germ cells, DAZL-positive cells are found in both adult animals and prepubescent mice. (F and H) show the overlay of a confocal and the corresponding differential interference contrast (DIC) image. While adult seminiferous tubules comprise premeiotic (DAZL-positive) and postmeiotic (DAZL-negative) germ cells surrounding a large lumen, P7 tubules are filled with DAZL-positive spermatogonia. (I-K) Confocal images of cultured cells isolated from seminiferous tubules at P7. Cultures were immunostained against DAZL. Nuclei were counterstained with DAPI. Virtually all DAPI-stained cells are also positive for DAZL. Note that cultures contain type A aligned spermatogonia. (L) SEM micrograph of cultured cells from P7 seminiferous tubules. Small round cells reside on top of a feeder layer of large, flat Sertoli cells.
Chapter 1. Introduction

1.3 Purinergic Signaling

1.3.1 Purinergic signaling mechanisms

Since adenosine 5’-triphosphate (ATP) has first been isolated from muscle tissue, ATP has been known as one of the biochemically most important cellular compounds. It is known as the major and universal cellular source of free energy, a function that was recognized first by Lohmann (1929). However, during the last 50 years, a general transmitter function of purines (including ATP) has been acknowledged as well. Drury and Szent-Györgyi (1929) observed the action of adenosine as a transmitter in the circulatory system and later Holton and Holton (1954) reported a transmitter function of ATP in dorsal root ganglia. The purine-induced effects were later linked to purinergic receptors (Burnstock, 1972).

Two different subtypes of purinergic receptors are distinguished: P1 and P2 receptors. The P1 family exclusively consists of guanine nucleotide-binding protein (G-protein) coupled receptors sensitive to adenosine (Ciruela et al., 2010). All members of the P2 family respond to ATP (Abbracchio and Burnstock, 1994). Within the P2 family there are both G-protein coupled receptors and ligand-gated ion-channels (Burnstock, 1997). The metabotropic receptors are called P2Y receptors (Abbracchio and Burnstock, 1994). In mammals, eight different members constitute the P2Y receptor family: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 (Burnstock, 2007). Extracellular ATP leads to P2Y-dependent activation of G-proteins. A typical signaling cascade – e.g., initiated by P2Y2 receptor activation – starts with GDP–GTP exchange at the trimeric G αq protein. Dissociated from the β/γ-complex, the GTP-bound α-subunit activates phospholipase C. The resulting cleavage of phosphatidyl inositol-4,5-bisphosphate into diacylglycerol and the cytosolic messenger inositol 1,4,5-triphosphate (IP3) triggers Ca2+ release from the endoplasmic reticulum by activation of IP3 receptors. Diacylglycerol can also activate signaling proteins, e.g. ion channels. The concept of G-protein coupled receptors allows activation of a multitude of different pathways.

By contrast, P2X proteins are ionotropic receptors (Friel and Bean, 1988; Abbracchio and Burnstock, 1994). Their activation gates an unselective cation permeability. P2X receptor activation at physiological resting membrane potentials results in membrane depolarization and activation of downstream voltage-dependent mechanisms (Sundelacruz et al., 2009; Franco et al., 2006; Wang, 2004). Additionally, P2X-mediated Ca2+ influx acts as a potent biochemical signal / second messenger.

Extracellular ATP is rapidly degraded by ubiquitous ectonucleotidases (Westfall et al.,
2000; Zimmermann, 2000; Robson et al., 2006). Ectonucleoside triphosphate diphosphohydrolase (E-NTPDase) thus represents an important regulatory factor in ATP signaling. This enzyme is membrane-bound and presents its catalytic domain to the cell’s exterior. E-NTPDase is responsible for the limited lifetime of an extracellular ATP signal. This allows precise signal modulation in time and space (Westfall et al., 2000; Zimmermann, 2000; Robson et al., 2006). Catabolic ATP degradation generates adenosine 5’-diphosphate (ADP) and adenosine 5’-monophosphate (AMP). These metabolites of ATP are degraded by ectonucleotide pyrophosphatase / phosphodiesterase to adenosine, which in turn acts on P1 purinergic receptors (Ziganshin et al., 1994). Adenosine is then degraded by the alkaline phosphatases to phosphate (Volonté and D’Ambrosi, 2009).

1.3.2 P2X Receptors

Three different families of ligand-gated ion channels can be distinguished: the P2X receptor-like family, the nicotinic receptor-like family and the glutamate receptor-like family (Khakh, 2001). According to their primary structure, those families have separate evolutionary origins (Green et al., 1998). The quaternary structure of P2X receptors is assembled from three subunits that form an unselective cation channel (Nicke et al., 1998; Barrera et al., 2005; Young et al., 2008; Kawate et al., 2009; Saul et al., 2013). Each monomer consists of two transmembrane domains which are connected by a large extracellular loop. The N- and C-termini are located in the cytosol (Eggan et al., 2004). The ATP binding side is located at the extracellular loop between two adjacent subunits. The second transmembrane domain primarily lines the channel pore (Kawate et al., 2009). When ATP binds to the receptor, the resulting conformational change leads to pore opening in an iris-like movement of the second transmembrane domain. (Hattori and Gouaux, 2012). There are seven different subunits (P2X₁₋₇), that can be expressed in heterologous systems (Khakh et al., 2001). The P2X receptor subunits show 39 - 55% sequence homology in their primary structure (North, 2002). There are also splice variants of P2X₁, P2X₂, P2X₄, P2X₅, P2X₆ and P2X₇ receptors, contributing to the diversity of P2XR signaling (Hardy et al., 2000; Simon et al., 1997; Townsend-Nicholson et al., 1999; Cox et al., 2001; Da Silva et al., 2007; Nicke et al., 2009; Adinolfi et al., 2010).

With the exception of P2X₆, all subunits can build homotrimeric channels (Lê et al., 1998). However, there is an ongoing discussion if P2X₇ is the only receptor that lacks heterotrimerization (Torres et al., 1999; Guo et al., 2007; Dubyak, 2007; Nicke, 2008; Saul et al., 2013). While early studies argue against heteromerization, more recent stud-
ies suggest a functional interaction of P2X₄ and P2X₇ receptors in a variety of tissues (Guo et al., 2007; Saul et al., 2013). Theoretically, the following heterotrimers were identified as possible combinations: P2X1/2, P2X1/3, P2X1/5, P2X1/6, P2X2/3, P2X2/5, P2X2/6, P2X3/5, P2X4/5, P2X4/6, and P2X5/6 by (Torres et al., 1999). Several of this heteromers were studied extensively while others lack functional description in heterologous assays (Saul et al., 2013). Homomeric receptors were described in a broad variety of tissues. P2X receptors differ in their ATP sensitivity. While the P2X₇ receptor is activated by ATP concentrations above 100\(\mu\)M, all other P2X receptors have their half maximal effective concentration (EC₅₀) between 1\(\mu\)M and 30\(\mu\)M (North, 2002). Moreover, the exact dose-response relationships for each channel vary in dependence of the expressing tissue and the composition of the extracellular solution (Virginio et al., 1999a; Riedel et al., 2007; Kubo et al., 2009; Li et al., 2013). Solely based on dose-response assays, it is not possible to distinguish between two P2X receptors with similar ATP sensitivity (e.g. P2X₂, P2X₆ and P2X₅).

The P2X-dependent current kinetics differ between several subunits. P2X₁ and P2X₃ show rapid desensitization whereas P2X₂ and P2X₄ desensitize more slowly. P2X₇ and P2X₅ are described as non-desensitizing (North and Barnard, 1997; Rettinger and Schmalzing, 2003; Coddou et al., 2011). At first activation, P2X₇ receptors show a biphasic current kinetic, that changes after prolonged or repetitive stimulation to a monophasic activation (Yan et al., 2008). The time course of this kinetics shift is correlated with the final plateau current (Chessell et al., 2001). Extensive activation of P2X₂, P2X₄ and P2X₇ can lead to increased membrane permeability, which ultimately allows passage of large molecules like YOPRO or N-methyl-d-glucamine (NMDG) (Surprenant et al., 1996; Virginio et al., 1999a; Rokic and Stojilovic, 2013). This phenomenon is widely referred to as pore-dilation (Virginio et al., 1999b). However, there is a controversial discussion about the mechanism causing the time-dependent change in conductance (Virginio et al., 1999a; North, 2002; Boldt et al., 2003; Browne et al., 2013; Stolz et al., 2015). Several groups discovered that P2X₂ and P2X₇ receptors instantly conduct NMDG during single channel recordings or in a symmetric whole-cell experiment (Riedel et al., 2007; Li et al., 2015). The gradually increasing conductance for large cations was never confirmed by single channel recordings. However, the P2X receptor pore shows massively decreased conductance when the current is driven by large cations. This indicates a preference for smaller cations (Ding and Sachs, 1999a; Riedel et al., 2007; Li et al., 2015). High membrane density of P2X₂ receptors might cause depletion and accumulation effects during patch-clamp experiments. Li et al. (2015) reported that these effects can occur when the
ATP-induced membrane conductance has a similar order of magnitude compared to the pipettes access conductance. Also the volume of the attached cell influences the effect. Ultimately, these effects lead to a massively increased current measured by the patch pipette. The recorded current kinetics show similarities to kinetics that were previously described as pore-dilation. However, many conflicting data exist and it is not yet clear that the recent findings can be confirmed in other laboratories.

### 1.3.3 Purinergic Signaling in the Testis

Purinergic signaling in the reproductive tract plays an important physiological role. So far, however, there are still many open questions about the influence of extracellular ATP in the testis. It was demonstrated that ATP is present in the extracellular environment of cultured Sertoli cells and that ATP release is increased by FSH application. In rat Sertoli cells, ectonucleotidase expression and activity was detected by both ATP degradation assays and immunohistochemistry. This activity was altered during rat Sertoli cell maturation and appeared to be stage specific (Barbacci et al., 1996; Casali et al., 2001, 2003; Martín-Satué et al., 2009).

The degradation of ATP ultimately leads to adenosine. The adenosine sensitive A\textsubscript{1} receptors were found in Leydig and Sertoli cells (Murphy and Snyder, 1981; Rommerts, 1984; Stiles et al., 1986; Monaco, 1986). Activating A1 receptors leads to a reduction in FSH-mediated adenylyl cyclase activity in Sertoli cells. Interestingly, the A1-mediated effect was exclusively observed at stages VII-VIII of the rat seminiferous cycle (Stiles et al., 1986; Monaco et al., 1988; Kangasniemi, 1993; Fredholm et al., 2001). In addition, several groups reported indications for FSH-induced autocrine ATP stimulation (Lalevée et al., 1999; Gelain et al., 2003, 2005).

Various groups found ATP-sensitive P2X receptors in the seminiferous epithelium. At first, P2X\textsubscript{4} and P2X\textsubscript{6} receptors were found in rat testis tissue using RT-PCR (Bo et al., 1995; Collo et al., 1996). The P2X\textsubscript{4} receptor was found in round spermatids and occasionally in immature sperm cells using \textit{in situ} RNA hybridization (Tanaka et al., 1996). Glass et al. (2001) found P2X\textsubscript{2}, P2X\textsubscript{3}, P2X\textsubscript{5} and P2X\textsubscript{7} in the adult rat testis using immunohistochemistry. These authors described a stage-dependent P2X expression pattern during the seminiferous cycle.

The first physiological investigation of P2 receptors were performed by (Filippini et al., 1994). They investigated cultured rat Sertoli cells using Ca\textsuperscript{2+} imaging and found indications for a UDP sensitive P2Y receptor. Other groups confirmed this finding using patch-clamp and Ca\textsuperscript{2+} imaging (Lalevée et al., 1999). However, there was physiological
indication that ATP leads to depolarization of Sertoli cells by Na\(^+\) influx. This might indicate an involvement of P2X receptors (Foresta et al., 1995). Veitinger et al. (2011) performed a detailed analysis of ATP-dependent signaling in cultured juvenile mouse Sertoli cells. They showed two separate mechanisms of ATP-induced Ca\(^{2+}\) influx. Using molecular, immunocytochemical and functional approaches, the authors of this study revealed the involvement of P2X\(_2\) and P2Y\(_2\) receptors in the Sertoli cells.

The inhibition of mitochondrial respiration suppresses germ cell apoptosis. This finding was connected to decreased ATP concentrations in the testicular tissue (Erkkila et al., 1999, 2003, 2006). It is possible, that P2X\(_7\) receptors are involved in the regulation of apoptosis. Hypothetically, activation of the P2X\(_7\) receptors could be impaired due to reduced ATP production (Burnstock, 2014). The P2X\(_7\) receptor is relatively insensitive to ATP. However, prolonged activation, results in a fatal Ca\(^{2+}\) influx. The involvement of P2X\(_7\) in apoptotic processes has been described in various (patho-) physiological contexts (Adinolfi et al., 2005).

Stage specificity of purinergic signaling has been reported several times, indicating its importance during sperm development (Kangasniemi, 1993; Glass et al., 2001; Casali et al., 2003; Burnstock, 2014). A recent review even pointed out the need to evaluate the role of germ cells in the purinergic signaling cascade (Burnstock, 2014). However, till today physiological experiments illuminating purinergic signaling in germ cells are still rare.

## 1.4 Tissue Clearing Techniques

The visualization of anatomical structures to understand their function is an essential method in life sciences. However, since most anatomical structures consist of tissue blocks with often many layers, merely examining the tissue surface is insufficient. In most cases, this issue is addressed by various methods of tissue slicing. This generates good accessibility for antibodies and other histochemical markers and allows acquisition of high-resolution images. However, reconstruction of the anatomic structure in its entirety is difficult.

To allow imaging of whole organs without tissue dissection, the penetration depth of light must be increased. Here, the major difficulty is to overcome the inert light scattering of biological tissue, hindering light penetration in deeper layers. About one century ago, Spalteholz, 1914 proposed a method to match the average refractive index (RI) of biological tissue with its mounting medium (Borlinghaus and Müller, 2014). The
method was performed using organic solvents with a RI of up to 1.5 (for comparison: air = 1; water = 1.33).

To highlight specific structures, fluorescence is commonly used for detection of primary antibodies or for expression profiling of fluorescent proteins in transgenic animals. However, organic solvent-based clearing methods lead to rapid quenching of fluorescence (Ke et al., 2013). Approaches of preserving the fluorophores within organic solvents for several days were established (Ertürk et al., 2012; Ertürk and Bradke, 2013). Moreover, aqueous solution-based clearing methods have been developed. These methods do not interfere with fluorescent molecules (Hama et al., 2011; Ke et al., 2013; Chung and Deisseroth, 2013; Chung et al., 2013). All these methods are based on a RI matching, comparable to the method first described by Spalteholz, 1914. However, even when the RI of the aqueous compartment is matched to the average RI of the protein and lipid fraction, there is still considerable RI variation. This leads to light scattering and limited spatial resolution during imaging (Cheong et al., 1990). Chung and Deisseroth (2013) presented a new method called CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/ Immunostaining/ in situ hybridization-compatible Tissue-hYdrogel) to overcome this problem. They immersed the samples in an acrylamide-tissue-hybrid. The lipid content was then removed from the specimen using detergents and an electric field. The tissue remained stable in the acrylamide matrix. This allows to match the RI of the tissue more precisely and increased penetration depth of light. The proposed solution for RI matching was Focus-Clear. However, this reagent is relatively expensive and its commercial availability is limited. Moreover, the RI of Focus-Clear is fixed to 1.454 (Tomer et al., 2014). RI matching solution (RIMS) was later introduced to adapt the CLARITY method to a wide variety of tissues (Yang et al., 2014).

Several groups established the method now. For example CLARITY was used to investigate the involvement of a cAMP response element-binding protein (CREB) overexpression in a neuronal subpopulation of the lateral amygdala in association to cocaine cue memory (Hsiang et al., 2014). CLARITY was also used to characterize hybrid periportal hepatocytes, which can regenerate liver injury without giving rise to cancer (Font-Burgada et al., 2015). This shows that CLARITY is a useful tool to provide new insight into complex tissue anatomy.
1.5 Aims

In males, spermatogenesis is a fundamental biological process that is critical for the survival of a species. Given the significance of spermatogenesis, it is surprising that only few details are known about testicular cell communication during this process. Since we and others have previously shown that Sertoli cells are able to communicate via ATP, I hypothesize a general role for purinergic signaling in the seminiferous cycle. Therefore, the overall aim of my thesis is to gain deeper insight into ATP-based signaling mechanisms in the murine seminiferous tubule.

Using wildtype C57BL/6 mouse pups, I will first establish a co-culture of Sertoli cells and spermatogonia. With this approach, I will be able to specifically analyze purinergic signaling in spermatogonia. First, I aim to utilize the whole-cell patch-clamp technique to investigate the functional expression of ATP-dependent ion channels in spermatogonia. The kinetic features of ATP-induced currents can shed light on the mechanistic and molecular basis of purinergic signaling. Adding pharmacological profiling as well as gene expression knockdown will allow the identification of the involved signaling proteins. I will use molecular biological, biochemical and Immunohistochemical methods to confirm my results.

Having analyzed the ATP-dependent signaling in cultured spermatogonia, I also aim to transfer this knowledge to a more complex in situ preparation. Therefore, I will first establish an acute tissue slice preparation from prepubescent mouse seminiferous tubules. Then, I will perform electrophysiological recordings from both Sertoli and germ cells and investigate the ATP-dependent signaling as well.

I will also participate in a collaborative project that is designed to investigate the function of the $\textit{GAR22}^{\beta}$ gene in the testes. Genetic ablation leads to infertility in male mice. I will investigate the expression pattern of the $\textit{GAR22}^{\beta}$ gene using a $\textit{GAR22}^{\beta}$ dependent $\beta$-galactosidase reporter expression approach. Additionally, I will investigate the colocalisation of $\textit{GAR22}^{\beta}$ with cell type-specific proteins using immunohistochemical methods. My results will provide an important contribution to the understanding of the role of $\textit{GAR22}^{\beta}$ in fertility.

As a third project, I will establish CLARITY, a method to render intact anatomical structures transparent. This will enable me to analyze whole organs (e.g. testes) or the intact brain in their entire complexity. New insights regarding connectivity and protein localization can be gained using this technique.

Together, the results gained during my thesis will provide deeper insight into the
complex physiology underlying cellular communication in the seminiferous tubule. My findings might allow the development of novel approaches to male contraception or, *vice versa*, infertility treatment.
Chapter 2

Materials and Methods

2.1 Material

2.1.1 Solutions

(a) **HEPES-buffered regular extracellular solution:**
145 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.3 (adjusted with NaOH); 300 mOsm (adjusted with glucose)

(b) **HEPES-buffered TEA extracellular solution:**
120 mM NaCl, 15 mM TEACl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, pH 7.3 (adjusted with NaOH); 300 mOsm (adjusted with glucose)

(c) **HEPES-buffered 110 nM free Ca²⁺ extracellular solution:**
145 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, 5 mM EGTA, pH 7.3 (adjusted with NaOH); 300 mOsm (adjusted with glucose)

(d) **Bicarbonate-buffered oxygenated (95% O₂/ 5% CO₂) extracellular solution:**
120 mM NaCl, 25 mM NaHCO₃, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 5 mM BES, pH 7.3 (adjusted with NaOH); 300 mOsm (adjusted with glucose)

(e) **Potassium based HEPES-buffered regular intracellular solution 110 nM free Ca²⁺:**
143 mM KCl, 10 mM HEPES, 1 mM EGTA, 2 mM KOH, 0.3 mM CaCl₂, 1 mM NaGTP, pH 7.1 (adjusted with KOH); 290 mOsm (adjusted with glucose)
(f) **Cesium based HEPES-buffered intracellular solution 110 nM free Ca²⁺:**
143 mM CsCl, 10 mM HEPES, 1 mM EGTA, 2 mM CsOH, 0.3 mM CaCl₂, 1 mM NaGTP, pH 7.1 (adjusted with CsOH); 290 mOsm (adjusted with glucose)

(g) **PCR-Mix-stock (1 ml):**
200 µl Buffer, 60 µl MgCl₂, 8 µl dNTP’s, 732 µl H₂O

(h) **RNA Lysis buffer (1 ml):**
10 µl βME, 990 µl buffer RLT Plus (provided in the Rneasy Plus Mini Kit)

(i) **TAE tris-acetate x50:**
2 M TRIS, 98 mM EDTA pH 8.0 (adjusted with ice cold acetic acid)

(j) **TRIS-Buffered saline with Tween 20 (TBST):**
0.605% TRIS, 0.8% NaCl, 0.1% Tween 20 in H₂O, pH 7.5 (adjusted with HCl)

(k) **Protein Lysis buffer:**
50 µl Triton X-100 and one tablet ’complete mini’ in 50 ml HEPES-buffered regular extracellular solution(a)

(l) **Laemmli buffer:**
20% glycerol, 4% SDS, 100 mM TRIS-HCl, and 0.2% bromphenol blue; pH = 6.8 (not adjusted)

(m) **Stacking gel:**
4.98% acrylamide/bis-acrylamide solution, 12.6% 1.5 M TRIS (pH 6.8), 0.1% SDS, 0.1% ammoniumpersulfate, 0.06% TEMED in distilled water

(n) **Separating gel:**
8.1% acrylamide/bis-acrylamide solution, 15% 1.5 M TRIS (pH 6.8), 0.1% SDS, 0.1% ammoniumpersulfate, 0.06% TEMED in distilled water

(o) **Transfer buffer:**
200 ml methanol, 2.9 g glycine, 5.8 g TRIS to 11 with distilled water

(p) **Blocking Solution:**
5% skim milk powder in TBST

(q) **Fixative solution:**
4% (w/v) PFA in PBS⁻/⁻
Chapter 2. Materials and Methods

(r) **Spermatogonia medium:**
epidermal growth factor 10 ng/ml, FBS 10%, FSH 1 ng/ml (7 mU/ml), growth hormone releasing factor 0.2 ng/ml, Insulin 5 μg/ml insulin-like growth factor-I 10 ng/ml, MEM non-essential amino acid solution 1%, nucleoside solution 0.01%, Penicillin-Streptomycin 100 U/ml, retinol acetate 5 μM, sodium pyruvate 0.5 mM, testosterone 100 nM, transferrin 5 μg/ml, in DMEM.

(s) **Washing Solution:**
10 mg/ml BSA in PBS⁻/⁻.

(t) **Blocking Solution:**
10 mg/ml BSA in PBS⁻/⁻, 5-20% normal goat serum, 0.02% sodium azide, 0.3% Triton X-100, supplemented by 5% (anti-P2X4, anti-P2X7, anti-BKCa antibody) or 10% anti-DAZL antibody.

(u) **Phosphate buffer 200 mM (PS):**
28 mM NaH₂PO₄, 171.9 mM Na₂HPO₄, pH 7.5 (adjusted with NaOH or HCl if necessary).

(v) **Hydrogel monomer solution (HM):**
4% acrylamide, 0.05% bis-acrylamide, 0.25% VA-044 Initiator, 4% PFA in PBS⁻/⁻.

(w) **Clearing Solution(CS):**
200 mM boric acid, 138 mM SDS, pH 8.5 (adjusted with NaOH).

(x) **PBS with Triton X (PBST):**
0.1% Triton X in PBS⁻/⁻.

(y) **Refractive index matching solution with 60% Nycodenz (nRIMS60):**
60 g Nycodenz, 20 mM PS(u), 0.1% Tween 20, 0.01% sodium acid.
### 2.1.2 Nucleic Acids

#### 2.1.2.1 Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>forward sequence</th>
<th>reverse sequence</th>
<th>annealing temperature in °C</th>
<th>Fragment size in bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X1</td>
<td>GAGAGTCGGCCAGGACTTC</td>
<td>GCGAATCCCAAACACCTTG</td>
<td>62</td>
<td>233</td>
</tr>
<tr>
<td>P2X2</td>
<td>TCCCTCCCCCACCTAGTCAC</td>
<td>CACCACCTGCTCAGTCAGGC</td>
<td>56</td>
<td>149</td>
</tr>
<tr>
<td>P2X3</td>
<td>CTTCTAACCTCAACCGACAAG</td>
<td>AATGCCCAGAAGCTCCACC</td>
<td>56</td>
<td>150</td>
</tr>
<tr>
<td>P2X4</td>
<td>CCCACTGCTGCCGAGATAT</td>
<td>CATATGCTTTGGGAGTGT</td>
<td>56</td>
<td>145</td>
</tr>
<tr>
<td>P2X5</td>
<td>GGAAGATAATGTTGAGGGTTGA</td>
<td>TCCTGATGAACCCCTCCAGT</td>
<td>54</td>
<td>81</td>
</tr>
<tr>
<td>P2X5b</td>
<td>GCCGCCCTCCACTGCAACC</td>
<td>AGGCCCCAGCCACAGATGAC</td>
<td>54</td>
<td>253</td>
</tr>
<tr>
<td>P2X6</td>
<td>TCCAGAGCATCCTTGGTTCCT</td>
<td>GGCACCAGCTCCAGATCTCA</td>
<td>54</td>
<td>152</td>
</tr>
<tr>
<td>P2X7</td>
<td>GCAAGAATTGTGGCACCAGTC</td>
<td>CCCACCCTCTGTCAGATTCT</td>
<td>55</td>
<td>171</td>
</tr>
<tr>
<td>P2X2_Splice</td>
<td>GACCTCCATCGGGGGTGGCT</td>
<td>ATGTCCTCGGGGAGCCAGCG</td>
<td>56</td>
<td>116 179 386</td>
</tr>
<tr>
<td>P2X7_Splice</td>
<td>GTCTCGCTACCGGGACACG</td>
<td>TGGGTCGCTGGAGTAGTGAGT</td>
<td>56</td>
<td>237</td>
</tr>
<tr>
<td>α1b-Tubulin (qPCR)</td>
<td>TCCCAAGATGTCAATGCTG</td>
<td>CACAGTGAGGCGCTGTAAT</td>
<td>58-63</td>
<td>115</td>
</tr>
<tr>
<td>P2X4 KD (qPCR)</td>
<td>TCCTGGCTTACGTCATTTG</td>
<td>AGAAGTGATGGGCTCGACAGCA</td>
<td>52</td>
<td>116</td>
</tr>
<tr>
<td>P2X7 KD (qPCR)</td>
<td>CCCAGATGGACTTCTCGGAC</td>
<td>GGACTTACGGGCGACACCTCTT</td>
<td>60</td>
<td>116</td>
</tr>
<tr>
<td>PCR Actin</td>
<td>GTATTCCCCTCAGTCGTTG</td>
<td>TGGATGCCACAGGATTC</td>
<td>56</td>
<td>750</td>
</tr>
</tbody>
</table>


### 2.1.2.2 Anti-sense RNA

Table 2.1: List of anti-sense RNA fragments used for P2X knock down experiments. All RNA fragments were purchased by Thermo Fisher Scientific

<table>
<thead>
<tr>
<th>RNAi Name</th>
<th>Thermo Fisher Scientific identifier</th>
<th>Order number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control #1, Antisense</td>
<td>4390843$AS00IZQ6</td>
<td>4390843</td>
</tr>
<tr>
<td>Control #2, Antisense</td>
<td>4390846$AS000TCK</td>
<td>4390846</td>
</tr>
<tr>
<td>siP2X2 (1)</td>
<td>4390771$AS00LTCV</td>
<td>s106892</td>
</tr>
<tr>
<td>siP2X2 (2)</td>
<td>4390771$AS00LTCU</td>
<td>s106893</td>
</tr>
<tr>
<td>siP2X4 (1)</td>
<td>4390771$AS00R7FE</td>
<td>s71184</td>
</tr>
<tr>
<td>siP2X4 (2)</td>
<td>4390771$AS00R7FD</td>
<td>s71185</td>
</tr>
<tr>
<td>siP2X7</td>
<td>4390771$AS00UBDN</td>
<td>s71189</td>
</tr>
</tbody>
</table>

### 2.1.3 Chemicals

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 488 sodium salt</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>2% Bis solution</td>
<td>Bio Rad</td>
</tr>
<tr>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)</td>
<td>AppliChem</td>
</tr>
<tr>
<td>4’,6-diamidino-2-phenylindole, dihydrochloride (DAPI)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>A 438079 hydrochloride</td>
<td>Tocris</td>
</tr>
<tr>
<td>Acrylamide solution</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Acrylamide/ bis-acrylamide (30% solution)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>ActinRed™ 555 ReadyProbes® reagent</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Agarose (low melting temperature)</td>
<td>PeqLab</td>
</tr>
<tr>
<td>Albumin from bovine serum (BSA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>AlexaFluor 488 goat anti rabbit secondary antibody</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>ATP disodium salt hydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>beta-Mercaptoethanol (βME)</td>
<td>AppliChem</td>
</tr>
<tr>
<td>BetaBlue™ staining kit</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>BLOCK-iT™ fluorescent oligo for lipid transfection</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>BlueJuice™ Gel loading buffer (10 x)</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

Boric acid Sigma-Aldrich
Bovine albumin VWR
Bromophenol blue sodium salt AppliChem
2’(3’)-O-(4-Benzoylbenzoyl) ATP (BzATP) Sigma-Aldrich
Ca\(^{2+}\) -Mg\(^{2+}\)-free phosphate-buffered saline tabs for 200 ml buffer (10 mM, pH 7.4) Sigma-Aldrich
Calcium chloride Sigma-Aldrich
Carbogen gas cylinder 50 l, ID-Gas:53 Westfalen AG
Cell mesh, nylon 70 μm Falcon / BD Biosciences
Cell scraper, 25 cm Sarstedt
Cesium chloride Sigma-Aldrich
Collagenase from *clostridium histolyticum* Sigma-Aldrich
Copper(I) chloride (Cu\(^{2+}\)) Sigma-Aldrich
cover glass, round 30 mm Assistent
cover glass, round 50 mm Life Technologies
CulturPlate-96 Perkin Elmer
D-glucose Sigma-Aldrich
Deoxynucleoside triphosphate (dNTP) mix Fermentas
Dimethyl sulfoxide (DMSO) Sigma-Aldrich
DirectPCR® Lysis Reagent Tail PeqLab
DRAQ5™ Fisher Scientific
Dulbecco’s phosphate-buffered saline (DPBS), Ca\(^{2+}\), Mg\(^{2+}\)-free Life Technologies
Dulbecco’s modified Eagle medium (DMEM) 1 g/l glucose + l-glutamine + pyruvate Life Technologies
Epidermal growth factor from murine submaxillary gland Sigma-Aldrich
Ethanol (EtOH) VWR
Ethylene diamine tetraacetic acid (EDTA) Sigma-Aldrich
Ethylene glycol tetraacetic acid (EGTA) Sigma-Aldrich
Extracellular anti-K\(_{Ca}\)1.1 (anti-BKCa) (APC-151) Alomone Labs
Extracellular anti-P2X4 receptor (APR-024) Alomone Labs
<table>
<thead>
<tr>
<th>Item</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracellular anti-P2X7 receptor (APR-008)</td>
<td>Alomone Labs</td>
</tr>
<tr>
<td>Extracellular DAZL (ab34139)</td>
<td>Abcam</td>
</tr>
<tr>
<td>Fetal bovine serum (FBS) &quot;superior&quot; charge: 1346W</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Focus Clear™</td>
<td>Tebu-bio</td>
</tr>
<tr>
<td>Follicle stimulating hormone (FSH)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Fura-2, acetoxymethylester (AM)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>GelRed™</td>
<td>VWR</td>
</tr>
<tr>
<td>GeneRuler 1 kb Plus</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Glass capillary, 1.05x1.5x100mm</td>
<td>Science Products</td>
</tr>
<tr>
<td>Glass capillary, 1.05x1.5x80mm</td>
<td>Science Products</td>
</tr>
<tr>
<td>Glutaraldehyde solution Grade II, 25% in water</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Glutaraldehyde (GA)</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Glycerol anhydrous</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Glycine</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Goat Serum</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>GoTaq® G2 Flexi DNA polymerase</td>
<td>Promega</td>
</tr>
<tr>
<td>Growth hormone releasing factor (human; synthetic)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Guanosine 5’-triphosphate (GTP) sodium salt hydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Hexamethyldisilazane (HMDS)</td>
<td>Merck Millipore</td>
</tr>
<tr>
<td>Histodenz™</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Iberiotoxin (IBTX)</td>
<td>Abcam</td>
</tr>
<tr>
<td>in vitro and in vivo dishes &amp; O-rings</td>
<td>Custom made</td>
</tr>
<tr>
<td>Insulin solution from bovine pancreas</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Insulin-like growth factor-I human</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Lipofectamine® 2000</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Magnesium chloride hexahydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methanol</td>
<td>VWR</td>
</tr>
<tr>
<td>Minimum essential media (MEM) non-essential amino acids solution, 100x</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES)</td>
<td>VWR</td>
</tr>
<tr>
<td>Item</td>
<td>Supplier</td>
</tr>
<tr>
<td>---------------------------------------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Nucleosides test mix</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Nycodenz™</td>
<td>PROGEN Biotechnik GmbH</td>
</tr>
<tr>
<td>Oligo(dt)23 primer</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Opti-MEM® I reduced serum medium</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Paraformaldehyde (PFA)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Penicillin-streptomycin (10,000 U/mL)</td>
<td>Life Technologies</td>
</tr>
<tr>
<td>Perfusion Pencil™</td>
<td>AutoMate Scientific</td>
</tr>
<tr>
<td>Petri dish, 100 mm, square-cut</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>Petri dish, 35x10 mm</td>
<td>Sarstedt</td>
</tr>
<tr>
<td>pH indicator strips, pH-Fix</td>
<td>VWR</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate</td>
<td>AppliChem</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Proteinase K-solution</td>
<td>VWR</td>
</tr>
<tr>
<td>PSB-POM144</td>
<td>PharmaCenter Bonn</td>
</tr>
<tr>
<td>PSB069</td>
<td>Tocris</td>
</tr>
<tr>
<td>QuantiTect® SYBR Green PCR kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Random hexamer primer</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Recording chamber Slice mini chamber</td>
<td>Luigs &amp; Neumann</td>
</tr>
<tr>
<td>Retinyl acetate water soluble</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>RevertAid™ H Minus M-MuLV reverse transcriptase</td>
<td>Fermentas</td>
</tr>
<tr>
<td>RNasin™</td>
<td>Fisher Scientific</td>
</tr>
<tr>
<td>Rneasy Plus Mini kit</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium phosphate dibasic dihydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium phosphate monobasic monohydrate</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Super glue</td>
<td>UHU</td>
</tr>
<tr>
<td>Super Silver razor blade</td>
<td>Gillette</td>
</tr>
</tbody>
</table>
Chapter 2. Materials and Methods

Suramin sodium salt
Tetraethylammonium (TEA) chloride
Tetramethylethylenediamin (TEMED)
Thiocarbohydrazide
Tissue Freezing Medium (TFM)
TRIS (hydroxymethyl) aminomethane
Triton® X-100
Trypsin-EDTA 0.05% (1X), phenol red
Tubing, silicone 6 mm x 9 mm
Tween® 20
Wako Va-044

Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Sigma-Aldrich
Leica Microsystems
VWR
Sigma-Aldrich
Gibco
VWR
AppliChem
Wako Chemicals

2.1.4 Equipment

**Equipment**

Lab power supply unit Voltcraft PS-302A
Binocular eyepiece S4E
BioPhotometer plus
Cell culture bench
Centrifuge mini spin
CO₂-incubator C150
Cryostat CM 1950
ECO Silver immersion thermostat
Hot plate magnetic stirrer
Hummer sputter coater
i-scan digitizer
iCycler IQ real-time PCR detection system
Microforge MF-830
Osmometer Osmomat 030
PC-10 Puller
PCR-Cycler Mastercycler ep gradient S
pH electrode InLab routine

**Supplier**

VoltCraft
Leica Microsystems
Eppendorf AG
Thermo Scientific
Eppendorf AG
BINDER
Leica Microsystems
LAUDA
VWR
Technics Inc.
ISS Group Services Ltd.
Bio-Rad
Narishige Group.
Gonotec
Narishige Group.
Eppendorf AG
Mettler Toledo
### Equipment

<table>
<thead>
<tr>
<th>Equipment</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH Meter five easy</td>
<td>Mettler Toledo</td>
</tr>
<tr>
<td>Thermomixer with Thermoblock</td>
<td>Eppendorf AG</td>
</tr>
<tr>
<td>VWR UnoCycler Thermal Cycler chassis with a 96 well gradient block</td>
<td>VWR</td>
</tr>
<tr>
<td>Water bath</td>
<td>Memmert</td>
</tr>
<tr>
<td>Water filter 5000l/h 2.54 cm (1Zoll) + polypropylene (PP)-Filter</td>
<td>Poly-Werkzeuge</td>
</tr>
</tbody>
</table>

### 2.1.5 Mouse strains

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>Charles River</td>
</tr>
<tr>
<td>OMP-GFP</td>
<td>kindly provided by Peter Mombaerts</td>
</tr>
<tr>
<td>GAR22β−/−</td>
<td>kindly provided by the Institute of Biomedical Engineering, Dept. of Cell Biology, Uniklinik RWTH Aachen</td>
</tr>
</tbody>
</table>

### 2.1.6 Setups

All microscopy setups were equipped with similar hard- and software for electrophysiology, micro manipulation and perfusion system.

For electrophysiology, a HEKA Elektronik EPC10USB or EPC10USB double was connected to a TIB14S trigger board to record, monitor and execute pulse protocols, respectively. The TIB14S trigger board was used to trigger application valves of a custom made application system for 8 separate solutions. The stimuli were applied using an 8-to-1 manifold connected to an application pencil (100µm or 250µm in diameter). The perfusion system allows stimulus switch with millisecond precision for instant liquid exchange in close proximity to the specimen. In addition, a bath perfusion was used to superfuse acute tissue slices constantly with fresh oxygenated extracellular solution.

The perfusion pencil, the patch-clamp electrode and for all upright setups, the shifting table were controlled by Luigs & Neumann micro manipulation.
Chapter 2. Materials and Methods

All microscopes were equipped for bright-field imaging using a Leica DFC360/5 FX CCD camera. For fluorescence imaging, an EL6000 light source was used. The microscopes were mounted on an optical table to minimize vibrations. For patch-clamp experiments, the microscopes were encapsulated by a Faraday cage to reduce electromagnetic noise.

2.1.6.1 Leica DMI4000 B

An inverse DMI4000 B microscope was used to investigate cultured cells. The setup is equipped for fluorescence imaging, including Fura-2 (fast switching external filter wheels), as well as phase contrast and bright-field imaging. The nosepiece is equipped with three objectives. The N PLAN 10x/0.25 PH1 (Leica) objective was used for low magnification imaging and positioning of the perfusion pencils. The HCX PL FL 20x/0.50 PH2 (Leica) objective was used for fluorescence imaging. The HCX PL FL L 63x/0.70 CORR PH2 (Leica) objective in combination with the camera (DFC360 FX CCD) was used during patch-clamp experiments. The I3 filter cube (Leica) was used for fluorescence detection of FITC labeled cells. For image acquisition the software LAS 2.X (Leica) was used.

2.1.6.2 Leica DM6000 FS

The upright DM6000 FS microscope was used to investigate acute tissue slices. This fixed stage setup, with micro manipulators that move relative to the stage and independent from the optical axes, provides freedom to position sample and probe while increasing overall stability. The microscope is equipped for fluorescence imaging, including Fura-2 (fast switching external filter wheels) as well as infra-red differential interference contrast (IR-DIC) and bright-field imaging. The camera (DFC365 FX CCD) is connected to a 3-position magnification changer (0.35 x - 1.25 x - 4.0 x). The nosepiece is equipped with two objectives. The HCX IRAPO L25x/0.95 W (Leica) objective was used for overview and to position the application at 0.35 x magnification and to perform patch-clamp experiments at 4.0 x magnification. The HCX APO L U-V-I 40x/0.80 (Leica) objective was used for Fura-2 imaging.

For Fura-2 imaging the FURA2 filter cube (Leica) was used together with external filters (387 nm/ 340 nm). The GFP ET filter cube (Leica) was used for fluorescence detection of Alexa 488 labeled cells. The patch-clamp amplifier is connected to a NI-DAQ board enabling synchronous sampling of electrophysiology data during Ca\textsuperscript{2+} imaging.
The standard image acquisition software was Leica LAS AF 3.0 to LAS X including the electrophysiology extension module.

2.1.6.3 Leica Confocal SP5

The upright Leica Confocal SP5 was used to investigate immunostainings. It is equipped for fluorescence imaging, DIC and bright-field as well as confocal imaging. The camera (DFC360 FX CCD) is connected to a 3-position magnification changer (0.35x - 1.25x - 4.0x). The microscope is equipped with the HCX APO L20x/1.00 W (Leica) objective.

The I3 filter cube (Leica) was used for fluorescence detection of GFP or Alexa 488 labeled cells.

For confocal imaging, five excitation laser lines were available: 458 nm, 476 nm, 488 nm, 514 nm, (argon laser); 633 nm (helium neon laser). Four dichroic mirrors (RT 30/70; TD 488/543/633; DD 458/514; RSP 500) were used to separate excitation from emission. For emission detection a SP detector was used. The SP detector allows distribution of emitted light to up to five distinct photomultipliers (three were used maximally). The spectral band with the shortest wavelength was directed to a hybrid detector (HyD) while longer wavelength bands were directed to standard PMT (photomultiplier) detectors. Additionally, one PMT was used to detect transmitted light to generate a scanned DIC image.

The standard image acquisition software was LAS AF 2 (Leica).

2.1.6.4 Leica Multiphoton SP8

An upright Leica SP8 microscope was used to investigate ‘CLARITY specimen’ (see: section 2.2.7). It is equipped for fluorescence imaging, IR-DIC and bright-field, as well as confocal and multiphoton imaging. The camera (DFC365 FX CCD) was connected to a 3-position magnification changer (0.35x - 1.25x - 4.0x). The microscope is equipped with the HC FLUOTAR L 25x/1.00 IMM (n_e=1.457) objective, which is optimized for high RI solutions and cleared tissues.

The I3 filter cube (Leica) was used for fluorescence detection of GFP labeled cells and the AT - EYFP/Venus/Citrine filter cube (Chroma) for Venus labeled cells.

For confocal imaging, five excitation laser lines are available (488 nm, 552 nm, 638 nm, by diode laser; 690 nm - 1040 nm by Mai Tai DeepSee). Three dichroic mirrors (RT 15/85; TD 488/552/638; DD 458/552) are used to separate excitation from emission light. A SP detector allows to distribute the emission light to up to five distinct photo detectors (maximally three were used). The spectral band with the longest wavelength
is directed to a HyD Leica while shorter wavelengths are directed to PMT detectors (Leica). Additionally, one PMT is used to detect transmitted light to generate a DIC image.

The standard image acquisition software was LAS AF 4.0 (Leica).

2.1.7 Software

<table>
<thead>
<tr>
<th>Software</th>
<th>Developer/ software house</th>
</tr>
</thead>
<tbody>
<tr>
<td>COREL Draw X6</td>
<td>Corel Corporation</td>
</tr>
<tr>
<td>Excel 2013</td>
<td>Microsoft</td>
</tr>
<tr>
<td>Fitmaster 2.67</td>
<td>Heka Elektronik</td>
</tr>
<tr>
<td>Gimp 2.7</td>
<td>The GIMP Development Team</td>
</tr>
<tr>
<td>Igor Pro 6.3.4</td>
<td>Wavemetrics</td>
</tr>
<tr>
<td>Imaris 8.1</td>
<td>Bitplane</td>
</tr>
<tr>
<td>Inkscape</td>
<td>inkscape.org</td>
</tr>
<tr>
<td>JPCalcW</td>
<td>Barry, 1994</td>
</tr>
<tr>
<td>Leica LAS AF/ LAS X</td>
<td>Leica Microsystems</td>
</tr>
<tr>
<td>Patchmaster 2.67</td>
<td>Heka Elektronik</td>
</tr>
<tr>
<td>Photoshop CS5</td>
<td>Adobe Corporation</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Spermatogonia Cell Culture

To achieve a primary spermatogonia/ Sertoli cell co-culture of high purity, juvenile male mice were decapitated at P7 and the testes were collected. The tunica albuginea was carefully opened with sharp forceps. Then the seminiferous tubules were squeezed out gently and transferred to 1ml MEM. To digest the tissue, 1ml collagenase in PBS/- (2mg/ml) was added and the mixture was gently swiveled at room temperature (RT) until the solution became cloudy (5 min). To stop the reaction 3ml serum-containing DMEM was added. Subsequently, the cell suspension was centrifuged for 3 min at 400 g.
The supernatant was discarded and the pellet was resuspended in 2 ml trypsin/EDTA. After 5 min incubation with gentle rocking at RT, the cell suspension was centrifuged for 3 min at 400 g. The resulting pellet was resuspended in spermatogonia medium(r) (1 ml/prepared testis). The cell suspension was transferred to 35 mm dishes (1 ml per dish) containing a 30 mm cover slip and 1 ml spermatogonia medium. After 24 h, 1 ml medium was added. After 48 h, one-third of medium was replaced by fresh medium every day. For the first three days in vitro (DIV), cells were incubated at 37 °C in a humidified incubator with 5% CO₂. From DIV4 to DIV7, cells were moved to a 34 °C humidified incubator with 5% CO₂ until they were used for patch-clamp experiments or molecular analysis. All media were heated to 37 °C prior to usage (Iwanami et al., 2006; Veitinger, 2009).

2.2.1.2 RNA-Interference (RNAi) Transfection of Cultured Spermatogonia

For small interfering RNA (siRNA) experiments cells were transfected between DIV3 and DIV6. Before transfection, confluency was verified. The culture medium was replaced by 2 ml penicillin/streptavidin-free medium. Cells were allowed to adapt (20 min, 37 °C in a humidified incubator with 5% CO₂). For each 35 mm dish, 250 μl Opti-MEM was mixed with 6 μl Lipofectamine 2000 and incubated for 5 min. siRNA was co-transfected with BLOCKiT fluorescent oligo (Fluorescein isothiocyanate (FITC) -labelled). Therefore, in a separate reaction tube 250 μl Opti-MEM was mixed with 6 μl siRNA (original concentration 6.25 μM; final concentration 15 nM in 2.5 ml) and 1.875 μl BLOCKiT fluorescent oligo (original concentration 20 μM; final concentration 15 nM in 2.5 ml) for each dish. Then, both batches were mixed gently and incubated for 30 min in darkness. Each 35 mm dish was supplemented with 500 μl of the solution and gently moved to distribute the transfection mix. Cells were used for experiments 12-24 h after transfection. Transfected cells were identified by FITC fluorescence using a Leica DMI 4000B inverse microscope. As negative control Silencer Select negative control no. 1 and 2 siRNA were used. Knockdown efficiency was checked by quantitative RT-PCR for each siRNA probe.

2.2.2 Molecular Methods

2.2.2.1 RNA Isolation of Cultured Germ Cells and Tissue Preparations
(Testis, Brain, Spinal Cord)

Total RNA from spermatogonia and organ tissue samples (testis, brain, spinal cord) were isolated according to the instructions of the RNeasy Plus Mini Kit. Lysis buffer
(h) was freshly prepared for each experiment.

For molecular analysis of cultured spermatogonia, DIV4 and DIV7 were used. To remove cultured spermatogonia from the Sertoli cell feeder layer, the medium was removed from the dish. Next, 1 ml PBS+/− per 35 mm dish was pipetted repetitively with moderate pressure directly onto the glass coverslip. Care was taken to avoid frothing. The supernatant of up to six 35 mm dishes was collected in a 15 ml centrifuge tube. Cells were washed two times by centrifuging at 400 g for 3 min and the supernatant was discarded. The pellet was re-suspended in 10 ml PBS+/−. Finally, cells were centrifuged at 400 g for 3 min and the pellet was re-suspended in 1 ml cold lysis buffer (h). Care was taken to complete the cell extraction procedure within less than 20 min. Cells were stored for up to one month until further processing (-80°C).

To homogenize cells or tissue extracts in lysis buffer (h), lysate was passed through a blunt 20-gauge needle. Persistent tissues were additionally sonicated for 10 min in an ultrasonic bath. The homogenized lysate was transferred to gDNA Eliminator spin columns and centrifuged (30 s; 17,000 g). The volume of the flow-through was supplemented with an equal volume of 70% EtOH and placed on an RNeasy spin column. The columns were centrifuged (15 s; 17,000 g), washed once with buffer RW1 and twice with buffer RPE (both provided in the kit). To elute RNA, 16.5 μl RNase-free water was pipetted on the spin column membrane. This step was repeated with the eluate to increase RNA concentration. Columns were centrifuged for 1 min at 17,000 g. RNA yield and purity was checked using a BioPhotometer plus. Samples with less than 1 μg/μl were excluded from further analysis. RNA extracts were directly used in RT-PCR.

2.2.2.2 RT-PCR of Cultured Germ Cell RNA and Tissue RNA Extracts
(testis, brain, spinal cord)

The reverse transcriptase reaction was performed with 5 μg extracted RNA. The RNA was mixed with the first reaction mixture consisting of 1.5 μM oligo(dt)23 primer, 1.5 μM random hexamer primer and 11.0 μl with RNase-free deionized water. The reaction Mix was incubated for 5 min at 65°C and directly stored on ice, followed by addition of the second incubation mixture consisting of 5 μl reaction buffer (5x), 1 μl (20 U) RNasin, 1 μl dNTP (25 mM) and 1 μl (200 U) RevertAidTM H Minus M-MuLV Reverse Transcriptase. This reaction mixture was incubated for 10 min at RT, followed by 60 min incubation at 42°C. The transcriptase was inactivated by heating (10 min; 70°C).

The resulting cDNA was used to perform a polymerase chain reaction (Mullis et al., 1986; Saiki et al., 1988). Therefore 50 μl PCR-Mix (g), 0.3 μl reverse primer (0.1 nM/μl),
0.25 µl GoTag Flexi polymerase and 0.5 µl-1 µl template cDNA (0.5 µg / 50 µl) were combined in a 200 µl PCR-Tube. The PCR was performed in a VWR UnoCycler using the PCR-protocol shown in table 2.6. The annealing temperature X was empirically determined and the elongation time was set to a value calculated from the expected fragment size and the speed of the polymerase (see section 2.1.2; GoTag Flexi Polymerase = 1 min/kb).

Table 2.6: PCR-Program: Standard PCR program illustrates time and temperature of PCR-steps. X is a variable for primer specific annealing temperatures that was empirically optimized for each primer pair

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>denaturation</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>annealing</td>
<td>X°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>elongation</td>
<td>72°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>final elongation</td>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

2.2.2.3 Gel Electrophoresis

Agarose gel electrophoresis was used to determine the PCR product size. GelRed™ (10,000x), was added to the hot 1% agarose gel to a 0.5x concentration. If the loading dye was not already provided within the PCR-Mix, samples were mixed with BlueJuice™ Gel Loading Buffer (10x) to a final concentration of 1x and loaded into the wells of the gel. The gel was then run in a gel chamber in 1x TAE (i) buffer at a voltage of approximately 10 V/cm. DNA was imaged in a VWR-GenoSmart gel documentation system under UV light (312 nm) with an 8 bit 768 x 582 pixel camera. To mark the size of a fragment, the GeneRuler 1 kb Plus DNA ladder was used.

2.2.2.4 Quantitative-PCR

Quantitative-PCR (qPCR) was performed with cDNA isolated from spermatogonia (see above 2.2.2.1). An amount of 25 ng cDNA was used according to the instructions of the QuantiTect SYBR Green PCR Kit. Briefly, 12.5 µl QuantiTect SYBR Green PCR Master Mix, 1 µl primer mix (0.1 nM/µl) and 1-5 µl DNA were all mixed in a reaction tube and filled with H₂O to a final volume of 25 µl. Amplification and detection of specific gene products were performed using an iCycler IQ (Bio-Rad) real-time PCR detection system. Threshold temperatures were selected automatically and all amplifications were followed by melting curve analysis. To check for identical primer efficiency, a template
dilution series was performed comparing the gene of interest primers and the reference gene primers efficiencies. Relative mRNA levels were calculated using the delta-delta cycle threshold (ΔΔCt) method (Livak and Schmittgen, 2001):

\[
\frac{X_{\text{test}}}{X_{\text{control}}} = 2^{\Delta\Delta C_T} = 2^{(C_{T,X} - C_{T,R})_{\text{control}} - (C_{T,X} - C_{T,R})_{\text{test}}}
\]

(2.1)

\(X_{\text{test}}\) relative expression of gene under test condition
\(X_{\text{control}}\) relative expression of gene under control condition
\(C_{T,X}\) threshold cycle gene of interest
\(C_{T,R}\) threshold cycle reference gene

Ct values were normalized to those of tubulin housekeeping genes (HKG). qPCR was performed in triplicate with testes from at least 18 animals.

2.2.3 Preparation of Vibratome Slices from Seminiferous Tubules

Male mice at P7 were sacrificed by decapitation using sharp surgical scissors. The testes were collected. The tunica albuginea was pinched open with sharp forceps and the seminiferous tubules were gently squeezed out and transferred into 1 ml PBS-. Tubules were carefully scattered and embedded in 4\% low-gelling temperature agarose. Then, the tubule / agarose block was cut into 200\,\mu m slices in oxygenated extracellular solution (d) at RT with a Leica VT1000S vibratome (speed: 0.15 mm/s; frequency: 65 Hz; amplitude: 1 mm). The acute slices were transferred to a storage chamber and submerged in constantly oxygenated extracellular solution at RT until use.

2.2.4 Electrophysiology

2.2.4.1 General Description of Whole-cell Patch-clamp Experiments

Electrophysiology essentially examines the electrical component of the electrochemical gradient that is present over biological membranes. Ion channels allow cells to make essential use of this gradient. Commonly, ion channels are gated in the course of various signaling cascades or allow cells to regain a physiological membrane potential rapidly.

The whole-cell patch-clamp technique is one of several methods to investigate ion channels in cell membranes. It was introduced by Erwin Neher and Bert Sakman between
Chapter 2. Materials and Methods

1973 and 1976 as an advancement of the conventional voltage-clamp technique. Key element of that innovation is the head stage (Neher and Sakmann, 1976; Hamill et al., 1981). The head stage is an amplifier positioned in a very close proximity to the probe. Therefore it is able to amplify currents in a picoampere range to a stable readout signal while maintaining a predetermined holding potential over the clamp. To record currents over the cell membrane, a fire polished patch pipette is sealed to the cell membrane. The seal resistance is important and must be at least in a gigaohm range. The pipette is filled with a solution based on typical intracellular ion concentrations. The membrane patch beneath the pipette tip is ruptured by a gentle suction pulse. The cytosol is rapidly replaced by the artificial intracellular solution. The seal resistance should remain unaltered, leading to strong noise reduction. This configuration allows the recording of currents over the whole cell membrane in the picoampere range.

2.2.4.2 Methodical Description of Whole-cell Patch-clamp Experiments

The amplifier was controlled by Patchmaster 2.60.0 to 2.73.4 software. If not otherwise stated, data were acquired with a gain of 10 mV/pF, filtered at 2.5 kHz by Bessel filters and sampled with (4 x filter factor) 10 kHz for digital recording.

The extracellular and intracellular solutions are balanced to perpetuate the electrochemical gradient. All solution pairs have symmetric chloride concentrations. Liquid junction potential correction was performed using Patchmaster’s build-in correction function. Liquid junction potential values were calculated by the software JPCalc for Windows (Barry and Lynch, 1991; Barry, 1994). The Ag/AgCl bath electrode was connected by a freshly-cut 150 mM KCl agar salt-bridge (in polyethylene tubing).

To establish the whole-cell patch-clamp configuration, fire-polished glass pipettes with a small tip (1-2 μm in diameter) were used. These pipettes were fabricated from borosilicate glass capillaries (with filament, 1.05 x 1.5x80 mm or 1.05 x 1.5 x 100 mm), which were pulled in two steps with a vertical micropipette puller (heat step 1: 62.3 %, drop length 4 mm; heat step 2: 46.9 %; 141 g). Pipette tips were heat-polished using a microforge (heat level: 50 %). Since pipette resistance is directly correlated to the pipette diameter, pipette resistance was used to monitor constant pipette characteristics. Pipettes with a pipette resistance of 5-6 MΩ ((a), (e)) were utilized for patch-clamp experiments.

To avoid clogging by floating debris, positive pressure was applied to the pipette before it entered the bathing solution. Moving closer to the target cell, positive pressure was decreased to a minimum to avoid cell damage. An automated offset correction of the patch-clamp amplifier was performed. The pipette was carefully moved towards the cell.
Chapter 2. Materials and Methods

until the remaining positive pressure dented the cell membrane. Instantly, a moderate negative pressure was applied to the patch pipette to attach the cell membrane to the patch pipette. The system was then allowed to build a gigaohm seal.

Automated $C_{\text{fast}}$ correction was achieved by using iterative software functions and executed when the seal resistance between the pipette and the cell was $> 1 \text{ G}\Omega$. Manual fine tuning of $\tau_{\text{pipette}}$ and $C_{\text{fast}}$ was applied to minimize capacitive currents.

Progressively increasing negative pressure pulses were applied to the patch pipette to rupture the membrane within the pipette. Automated $C_{\text{slow}}$ correction was applied after the whole-cell configuration was established using iterative software functions and manual fine tuning. Resulting $C_{\text{slow}}$ values correspond to the surface area of the cell membrane and were used to calculate the current density (pF/pA) of individual cells. Test pulses were used between all pulse protocols to test for access resistance changes.

2.2.4.3 Electrophysiological Recordings of Cultured Spermatogonia

Cultured spermatogonia were patched using an inverted microscope (2.1.6.1). The 30 mm cover slips were removed from the culture dish and transferred to the "in vitro and in vivo dish" (2.2). Cells were washed in extracellular solution (a) and after controlling the dish for leakage, it was transferred onto the microscope stage.

2.2.4.4 Electrophysiological Recordings of Acute Tissue Slices from Seminiferous Tubules

Vibratome slices (200\(\mu\)m) of seminiferous tubules isolated from juvenile mice were transferred to a recording chamber. Tubules were continuously superfused with fresh oxygenated extracellular solution (d). Using the Leica DM6000 FS microscope, cells within cross-sectioned tubules were patched. The cytosol of the patched cell was loaded with a fluorescent dye (Alexa 488; 20\(\mu\)M) by the patch-pipette, to allow morphological identification.

2.2.5 Immunohistology

2.2.5.1 Cryosections and Immunostainings of Testis Tissue

For immunostainings of testicular cryosections, testes were fixed with 4\% PFA in PBS\(\sim\) (q) (overnight; 4°C) followed by cryoprotection in PBS\(\sim\) containing 30\% sucrose (\(\geq 24\)h; 4°C). Testes were embedded in TFM and sectioned at 20\(\mu\)m on a cryostat (-22°C). The
sections were incubated in blocking solution (t) (1 h, RT). Sections were washed (2 x 5 min in washing solution (s)), followed by the incubation with the respective primary antibody (1:500 in (s), overnight, 4°C, in a humidified chamber). Subsequently, sections were washed (5 x 10 min (s)), followed by incubation with AlexaFluor 488 secondary antibody (1:500 in (s); 1 h; RT). Finally, they were washed to remove unbound secondary antibody (3 x 5 min (s), 2 x 5 min PBS/>.

### 2.2.5.2 Immunoblotting

For immunoblotting several positive/negative control tissue samples (brain, olfactory bulbs, muscle and HEK293T cells) were collected and kept at -20°C until use. After adding 100 μl of protein lysis buffer (k) and homogenizing (micro pestle or sonication for 5 s), samples were centrifuged (10 min; 1000 g; 4°C). Protein concentrations of the supernatant were determined photometrically and adjusted to 1.5 mg/ml by adding of lysis buffer (k). The probes were diluted in a 1:1 ratio with Laemmli buffer (l). Polypeptide chains were denatured by heat incubation (5 min; 95°C). Gel electrophoresis equipment was filled with separating gel (n). After 30 min the stacking gel (m) and the comb were inserted. The gel slots were filled with 3 μl of marker (PageRuler Plus Prestained Protein Ladder) and 15 μl of boiled protein probe. Electrophoresis was run at 80 V (4°C), until the proteins reached the separating gel. Subsequently, voltage was increased to 120-150 V (1 h). For Western Blot all components (sponges, Whatman paper and membrane) were pre-soaked in transfer buffer (o). After blotting (25 min; 100 V), the membrane was rinsed in aqua dest. and blocked in Blocking Solution (p) (overnight; 4°C; under agitation). After blocking, the membrane was incubated with 2.5% skim milk powder/TBS-T solution containing the primary antibody at the desired concentration (1 h; RT). The membrane was washed in TBS-T (j) (4 x 15 min) and rinsed in 2.5% skim milk powder/TBS-T solution containing the secondary HRP-conjugated antibody (1:5000; RT; 45 min; under agitation). Membranes were incubated with Lumi-Light Western blotting substrate (5 min) and exposed to a photo film.

### 2.2.6 Data Analysis

Every experiment was performed at least on two different days (usually more) using different application paradigms each day. Individual numbers of experiments (n) are provided in figure legends. If not stated otherwise, data is presented as means ± SEM and statistical analyses were performed using paired, unpaired t-Tests or ANOVA /
Tukey test (as dictated by data distribution and experimental design). A p-value of ≤ 0.05 was considered significant. Statistical tests were performed using Excel and Igor-Pro. Unless otherwise stated, all experiments were performed by myself. I used the "we" form in this thesis to express my gratitude for excellent supervision and great support by all lab members.

2.2.7 CLARITY

2.2.7.1 Whole Animal Perfusion Fixation for Rodents

HM (v) was thawed in the refrigerator one day prior to the experiment. Adult mice were sacrificed using gradually increasing CO_2 levels. Maximum CO_2 levels were maintained for at least 2 min after respiration terminated. The toe pinch-response method was used to determine depth of anesthesia. A lateral incision through the integument and abdominal wall was introduced just beneath the rib cage. The diaphragm was cut along the entire length of the rib cage to expose the pleural cavity. Two cuts along the side of the ribs were taken, carefully displacing the lungs, up to the collar bone. A 15-gauge blunt tipped perfusion needle was pierced through the left ventricle into the ascending aorta until the tip was visible through the wall of the aorta. A hemostat was used to keep the needle in place during the perfusion. Finally, a small incision was made in the right atrium to allow blood release.

The outlet port of the de-aired perfusion apparatus was attached to the needle and 50 ml of cold PBS/- was pumped into to the vascular system with a pressure of 120-80 mmHg using a manometer bulb. When the liver was cleared of blood the buffer valve was switched to fixative release. At a constant flow rate, 20 ml of HM were released into the vascular system. Organs were dissected and stored for three to four days in HM for further fixation (4°C).

2.2.7.2 Hydrogel Tissue Embedding

To facilitate hydrogel polymerization, O_2 was removed from the HM (v) by degassing. Tubes containing samples (50 ml or 15 ml conical tube) were filled up to one third of their total volume to avoid liquid spilling during the degassing process.

Samples were degassed twice using a desiccator. The tubes were put into a desiccation chamber and opened to the extent that allowed to allow gas exchange. A vacuum pump was connected to the desiccator. After several minutes in vacuum, bubbles were visible in the tubes. After 20 min, the desiccator was sealed, the vacuum pump was disconnected
Chapter 2. Materials and Methods

and a nitrogen or argon tank was connected to the chamber. Oxygen free gas was released into the desiccator until atmospheric pressure was re-established. Then, the procedure was repeated.

After the second degassing step, the desiccator hood was removed just enough to reach the tubes. Quickly the caps of the tubes were tightened to maintain an oxygen free atmosphere in the tube.

The tubes were incubated for 3 h at 37°C until the solution polymerized.

The embedded samples were extracted. Extra gel pieces were removed and the samples were washed once at RT for 24 h and twice at 37°C for 24 h in clearing solution to remove residual PFA and monomer.

2.2.7.3 Electrophoretic Tissue Clearing (ETC)

The CLARITY setup is placed under a fume hood because many hazardous substances were used in relatively high concentrations during the clearing process.

The sample was moved to a custom-made specimen enclosure that allows liquid exchange. It was placed into the ETC chamber. For fluid circulation and temperature regulation, a LAUDA ECO Silver immersion thermostat was immersed in a 3 l bath and connected to the ETC. The whole setup required 4.5 l of clearing solution(w) to circulate. The clearing buffer was filtered by a water filter (50001/h & polypropylene PP-filter) before entering in parallel connected electrophoretic tissue clearing (ETC) chambers. Clearing solution was circulated through the chambers at moderate flow rate while heating the solution to 37°C. Three Voltcraft PS-302A power supply units were used to provide electricity for up to six ETC chambers. Chambers with the same voltage settings were connected in parallel until the maximum current output of the power supply (2 A) was reached. The ETCs electrodes were electrically powered to 10-30 V for 2-12 days. After clearing, the samples were washed twice for 24 h in PBST (x).

2.2.7.4 Imaging of Cleared Samples

Before imaging the sample it was incubated for one day in nRIMS (y).

Nuclei staining was applied in PBST or nRIMS using DAPI, DRAQ5 or PI, the incubation time was at least one day (up to five days). For imaging, a Leica Multiphoton SP8 confocal microscope or a Zeiss Lightsheet Z.1 with a 20x objective was used.
2.2.8 Scanning Electron Microscopy

Freshly prepared seminiferous tubules were transferred to 100% pure EtOH, cooled by nitrogen. Freeze fractures were established by a sharp scalpel after the samples were completely frozen. Fractured samples were transferred to -80°C cold EtOH containing 0.5% GA and stored for 48 h. GA concentration was consecutively increased by 2% GA (48 h, -80°C), 4% GA (96 h, -20°C) and 10% GA (120 h, 4°C). After washing in HMDS for subsequent air drying (RT), the specimens were gold sputter coated to a thickness of up to 2 nm (8 min, 10 mA sputter current). SEM images were acquired using a Cambridge Stereoscan S604 SEM connected to an i-scan digitizer (ISS Group Services Ltd., Manchester, UK). (Adapted from dissertation Ingo Scholz)
Chapter 3

Results

3.1 Ion Channels in Spermatogonia

3.1.1 Electrophysiological Characterization of ATP-induced Responses in Prepubescent Spermatogonia

Extracellular ATP is used as a signaling molecule in many tissues (Foresta et al., 1995; Veitinger et al., 2011). To investigate if spermatogonia respond to increased extracellular ATP concentrations we utilized the whole-cell patch-clamp technique (figure 3.1; B). We analyzed the response of these cells to different extracellular ATP concentrations and different stimulation times. A little more than half of the spermatogonia responded to the ATP stimulation. The proportion of ATP sensitive cells remained stable (~ 60%) during the culture time with a small increase at DIV 7 (~ 80%) (figure 3.1; A). (figure 3.2; A). Application of a low ATP concentration (30μM) at a negative holding potential resulted in rather fast developing inward currents. Increasing the application time also increased the maximal current amplitude with current saturation at 1s ATP stimulation. Longer exposure induced a moderate current decline in the presence of the stimulus (figure 3.2; A and B).

We also tested the effect of changing the interstimulus interval (ISI) (figure 3.3 3). An ISI of at least 46s is necessary to prevent current desensitization and was chosen for all future experiments.

The ATP-induced current increased dose-dependently and reached saturation between 100μM and 300μM with an EC50 value of 13.5μM. Interestingly, raising the ATP concentration further lead to a dramatic increase of the current amplitude resulting in a double dose-response curve (figure 3.4 A and B). The pH-values for ATP concentrations
Figure 3.1: Response rate of cultured spermatogonia: (A) Bar chart showing the percentage of ATP-sensitive cells (stimulation 10 – 100 μM) over the DIV. The number of cells tested is indicated above individual bars. At DIV7 the number of ATP sensitive cells increased slightly (t-Test p = 0.02). (B) Phase-contrast micrograph depicting single, double and aligned spermatogonia on top of a Sertoli cell layer. The single spermatogonium is targeted by the patch pipette.

> 1 mM were adjusted with NaOH.

3.1.2 Varying the Membrane Potential Reveals two Different ATP-induced Responses.

To analyze the ATP-induced current in more detail, we recorded ATP-induced currents at different holding potentials. Varying the holding potentials revealed two ATP-induced currents. At negative membrane potentials ATP induced the instantaneously developing inward current ($I_{\text{ins}}$). This current showed strong inward rectification. Additionally, we observed a delayed outward current that was absent at -80 mV but increased at less negative and positive potentials (figure 3.5).

We first investigated the nature of this ATP-induced delayed current component. We observed this current in ~ 40% of the cells under control conditions(e). Replacing intracellular K+ ions by Cs+ (f) abolished the delayed outward current completely (figure 3.7 B). This suggests the involvement of potassium channels as most potassium channels are impermeable for Cs+.

Using a pharmacological approach we tried to manipulate selectively the delayed com-
Chapter 3. Results

Figure 3.2: Extracellular ATP induces currents in prepubescent spermatogonia. (A) Representative original whole-cell current recording showing the response of a spermatogonium stimulated with extracellular ATP (30 μM) at a holding potential of -80 mV. Increasing the stimulation time revealed an increase in response amplitude as well as moderate desensitization of the ATP-induced current in presence of the ligand. (B) Quantification of data shown in (A). 1 s ATP stimulation is sufficient to elicit the maximal current amplitude. This current is significantly larger than the current elicited by a 0.3 s stimulation, Test p = 0.01. Data are plotted as mean ± SEM. Numbers of experiments are indicated above bars.

Figure 3.3: Determining the appropriate ISI: The ISI affects the mean maximal current amplitude during repetitive stimulation (V\text{hold} = -80 mV, 100 μM ATP, n = 8). The inset shows a representative recording, ATP Stimulation (200 ms each) is indicated above the trace.
Figure 3.4: Dose-response relationship for ATP: (A) Original traces of two cells challenged with different ATP concentrations. (B) Quantification of data shown in (A). Maximal ATP-induced currents were normalized to the response induced by 100 μM ATP. Shown is the mean ± s.e.m., n = 5 – 50. Data were fitted using the Hill Equation. Fitting the data points up to 300 μM ATP (shown in black) results in an EC50 of 13.5 ± 1 μM and a Hill coefficient of 1.6 ± 0.2.
Figure 3.5: Current-Voltage (I-V) relationship of ATP-induced currents: (A) Original recording showing the ATP-induced currents at different holding potentials (indicated next to each trace). An ATP-induced inward current reaches its maximum during the stimulation (instantaneous current, $I_{\text{ins}}$). An outward current appears later and reaches its maximum after the ATP stimulation (delayed current, $I_{\text{del}}$, blue coloring denotes area considered for current analysis). (B) I-V relationship of the ATP-induced currents shown in (A). Maximal current amplitudes were measured during the ATP application ($I_{\text{ins}}$) or during the time frame marked blue ($I_{\text{del}}$). To calculate the current density ($\text{pA/pF}$) current amplitudes were divided by the measured membrane capacitance. Data are mean ± s.e.m., $n = 18$, ISI = 180 s, 100 μM ATP. $I_{\text{ins}}$ (white dots) shows strong inward rectification with almost no current at positive potentials. $I_{\text{del}}$ (blue dots) has its reversal potential at -80 mV and increases continuously at all measured potentials.
Figure 3.6: Replacement of intracellular K⁺ affects delayed current (A) Original recording of the ATP-induced currents at different holding potentials using Cs⁺-based pipette solution. The amplitude of the instantaneous current was measured during the ATP application. The time frame for the measurement of the delayed current amplitude is marked blue. Data are mean ± s.e.m., n = 6, ISI = 180 s, 10 μM ATP. K⁺ was replaced by Cs⁺ in the intracellular/pipette solution ((f)). (B) I-V relationship of ATP-induced currents.
Figure 3.7: Comparison of current occurrence: (A) Bar chart showing the percentage of cells showing the ATP-induced instantaneous current (stimulation 10 – 100 μM) over the DIV with control (e) and Cs²⁺-based (f) intracellular solution. The number of cells is indicated above individual bars. (B) Bar chart displaying the percentage of ATP-sensitive cells showing the delayed outward current. Under control conditions (K⁺-based pipette solution (e)) 18% (DIV4), 36% (DIV5), 42% (DIV6) and 44% (DIV7) of the cells displayed the delayed outward current. However, this current never occurred when intracellular K⁺ was replaced by Cs⁺.
ponent of the ATP-induced response. First, we used TEA, a well characterized open
cbannel blocker affecting a variety of potassium channels (Shuster and Siegelbaum, 1987).
Strong inhibition by extracellular TEA (15 mM; (b)) confirmed our hypothesis. We re-
duced extracellular Ca^{2+} to test if the delayed current component is triggered by Ca^{2+}
influx. Interestingly, when extracellular Ca^{2+} was dramatically reduced (from 2 mM
to 110 nM), the instantaneous ATP-induced inward current was significantly potenti-
ated now probably carried mainly by Na^{+} ions) while the delayed outward current was
strongly diminished. This shows that the activation of the delayed outward current
depends on the influx of extracellular Ca^{2+}. IBTX, a specific blocker for large conduc-
tance Ca^{2+}-dependent potassium (BKCa) channels (Yellen, 1984; Galvez et al., 1990),
also strongly inhibited the delayed current without influencing the instantaneous current
and thus identifies the delayed activated channels as BKCa channels (figure 3.8).

We also investigated how ATP stimulation affects the spermatogonial membrane po-
tential (figure 3.9). Using the current-clamp mode in the whole-cell patch-clamp con-
figuration, we measured a resting membrane potential of \(-36 \pm 5\) mV \((n = 19)\) without
current injection. Application of ATP quickly depolarized the membrane potential to \(+20\) mV, followed by a slower developing hyperpolarization to \(-55\) mV. The hyperpo-
larization occurred after the ATP stimulation stopped. Then the membrane potential
slowly returned to the resting membrane potential. In the following experiments we
used the Cs^{+}-based pipette solution (f) to prevent the development of the delayed cur-
rent conducted by BKCa channels, unless otherwise stated.

### 3.1.3 Molecular Analysis of P2X Receptors in the Testicular Tissue

We next aimed to identify the ATP-dependent channels conducting the instantaneous
inward current. Ionotropic P2X receptors are good candidates as they are directly acti-
vated by ATP and also conduct Ca^{2+}. To investigate the expression of P2X receptors, we
first designed specific intron-spanning primers for the different P2rx genes and performed
RT-PCR experiments on whole RNA extracted from primary mouse spermatogonial cell
cultures, immature testis (P7), and whole brain–spinal cord tissue (positive control; 
Burnstock, 2008). We amplified cDNA encoding P2X_{2}, P2X_{4} and P2X_{7} receptors from
cultured cells as well as immature testes (figure 3.10).

A faint band encoding P2X_{3} was observed in immature testes but not in cultured
cells. None of the remaining P2rx genes appears to be expressed in mouse testes. We
Figure 3.8: Pharmacological profile of the delayed outward current. (A) Original whole-cell voltage-clamp traces, \( V_{\text{hold}} \) is indicated next to the trace. ATP was 30 \( \mu \text{M} \) (TEA and reduced \( \text{Ca}^{2+} \)) or 1 mM (IBTX). Preincubation (2 min, respectively) with 15 mM TEA (b) (upper trace) reduced \( \text{Ca}^{2+} \) solution (110 nM \( \text{Ca}^{2+} \), middle trace) or 100 nM IBTX (lower trace) abolished the delayed outward current. (B) Quantification of the data shown in (A). Data are mean ± s.e.m., the number of cells tested is indicated above individual bars and paired Student’s t-Test was used to test for significance. TEA significantly reduced the delayed outward current, \( p = 0.0009 \). Reducing the extracellular \( \text{Ca}^{2+} \) concentration significantly increased the ATP-induced instantaneous inward current, \( p = 0.00002 \). 10 of these 31 ATP-sensitive cells showed a delayed outward current which was significantly diminished by reducing extracellular \( \text{Ca}^{2+} \), \( p = 0.00002 \). IBTX also significantly inhibited the delayed outward current under control conditions, \( p = 0.01 \).
**Figure 3.9: ATP affects the membrane potential.** (A) Original whole-cell current-clamp recording. ATP (100 μM) induced a strong depolarization that reached a plateau during the stimulation. After the stimulation the membrane potential repolarized and even hyperpolarized to values more negative than the resting membrane potential. Then, the membrane potential slowly returned to the resting membrane potential. (B) Quantification of data is shown in (A). Data are mean ± s.e.m. For better comparability in some cells the membrane potential (without ATP) was adjusted to the resting membrane potential by a small constant current injection.
Figure 3.10: RT – PCR reveals P2Xr transcripts. We observed transcripts for P2xr2, P2xr4 and P2xr7 in the spermatogonial culture as well as in whole testis samples (P7). A faint band encoding P2X3 was detected only in whole testis. The expected PCR products were 150 – 250 bp, respectively. cDNA from whole brain / spinal cord was used as positive control.

recently described the functional expression of P2X2 receptors in mouse Sertoli cells (Veitinger et al. (2011)). Thus it is possible, that either only Sertoli cells or Sertoli cells and spermatogonia express P2X2 receptors. While P2X2 and P2X4 receptors display a high ATP sensitivity of a similar level in the low μM range, P2X7 receptors are rather ATP insensitive and need much higher concentrations to be activated.

Immunohistochemical localization of P2X4, P2X7 receptors and K_{Ca}1.1 channels in testicular sections showed broad expression pattern. Therefore, it is not possible to identify the expression in the different cell types (figure 3.11). Control experiments omitting the primary antibodies showed only a sparse background signal by the fluorescence labeled 2nd antibody. A peptide control was carried out to verify the first antibody’s specificity to the binding site of the target protein. After preincubation with the control peptide, the antibody showed reduced signal in the immunostaining. The specificity of the primary antibody was additionally assessed with western blot experiments. Lysates from brain, olfactory bulbs, muscle and HEK293T cells were used as positive/negative controls. Additionally, the blotting experiment was carried out with antibodies that were preincubated with the control peptide. This treatment eliminated the positive signals detected in tissue from seminiferous tubules and the positive control tissues, respectively.
Figure 3.11: Immunostainings and Western blots
Figure 3.11: Immunostainings and Western blot analysis: Anti-P2X4, anti-P2X7 and anti-KCa1.1 antibody staining in cryosections of juvenile seminiferous tubules reveals a wide distribution through the whole tubules. The first row shows the fluorescence signal only. The second row shows an overlay of the fluorescence with the corresponding DIC image. (A - C) Anti-P2X4 receptor staining (A), control staining in the absence of the primary antibody (B) and staining after preincubation with peptide control (C), n=3. (E – G) Anti-P2X7 receptor staining (E), control staining in the absence of the primary antibody (F) and staining after preincubation with peptide control (G), n=3. (I – K) Anti-BCa1.1 channel staining (I), control staining in the absence of the primary antibody (J) and staining after preincubation with peptide control (K), n=3. (D, H, L) Corresponding Western blots using the respective antibody (top panel). The bottom panel shows the control blot after preincubation with the respective peptides. (D) Anti-P2X4 Western blot with and without peptide control. (1: muscle; 2: adult testis, 3: P7 testis, 4: kidney, 5: brain); (H) Anti-P2X7 Western blot with and without peptide control. (1: HEK-293 cells, 2: Brain, 3: P7 testis, 4: adult testis); (L) Anti-KCa1.1 western blot with and without peptide control (1: HEK-293 cells, 2: Brain, 3: P7 testis, 4: adult testis).

3.1.4 Identifying the channels activated by low ATP concentrations

Having identified P2X2, P2X4 and P2X7 receptors as possible ATP sensitive channel candidates on the molecular level, we aimed to identify which of these channels are functional in spermatogonia using whole-cell patch-clamp combined with pharmacological tools. First, we focused on the channels activated by low ATP concentrations. Therefore, we used the inhibitor suramin which inhibits P2X2 (and other P2X receptors), but not (or only slightly at high concentrations) P2X4 or P2X7 receptors (Townsend-Nicholson et al., 1999; Jones et al., 2000; Donnelly-Roberts et al., 2009). The instantaneous inward current activated by a low ATP concentration (10\text{M}) in spermatogonia was not inhibited by suramin (3.12). We also investigated the suramin effect on ATP-induced currents in Sertoli cells, which are known to be mediated by P2X2 receptors (Veitinger et al., 2011). Indeed, this current was dose-dependently inhibited by suramin (figure 3.13), proving the efficacy of the drug.

When spermatogonia where challenged with low doses of ATP applied in the presence of Cu$^{2+}$ or under acidic conditions (pH 6.3), the resulting current was significantly reduced (figure 3.12). This is typical for P2X4 but not for P2X2 receptors (Xiong et al., 1999; Acuna-Castillo et al., 2000; Stoop et al., 1997; King et al., 1996). Finally, we tested the specific P2X4 receptor modulator ivermectin (Sim, 2006) which signifi-
cantly potentiated the ATP-induced current (figure 3.12). These results suggest that low ATP-induced currents are conducted by P2X$_4$ receptors, while P2X$_2$ receptors are not functionally expressed in spermatogonia.

To confirm the functional expression of P2X$_4$ receptors in spermatogonia, we performed RNAi experiments. Effective P2X$_4$ gene silencing (knockdown) by transient transfection with small interfering RNAs (siRNA) was controlled by quantitative real-time PCR (figure 3.14 A). Relative to controls, P2X$_4$ transcript levels were significantly reduced to 47 ± 4% (RNAi 1) and 35 ± 5% (RNAi 2), respectively, while the non-targeting negative control siRNA had no significant effect. To identify transfected cells for electrophysiological experiments, we co-transfected each siRNA construct with a fluorescent marker (figure 3.14 B).

Down regulation of P2X$_4$ gene expression with either of the siRNAs led to significant reduction of the ATP-induced current compared to non-transfected cells as well as non-targeting siRNA controls (3.15). We also tested the effect of P2X$_2$ receptor gene knockdown. While we observed no significant current reduction in spermatogonia (3.15), the ATP-induced current in transfected Sertoli cells was strongly inhibited (figure 3.16). Together, these results confirm that spermatogonia functionally express P2X$_4$ but not P2X$_2$ receptors.

### 3.1.5 High ATP Concentrations Activate Additional Low Sensitivity ATP Receptors

In the ATP dose-response curve we observed an additional increase in current amplitude at ATP concentrations ≥ 1 mM figure 3.4. Our PCR experiments showed the expression of P2X$_7$ receptors3.10 which are known for their low ATP sensitivity. To test whether these receptors mediate the additional current increase in spermatogonia, we compared the currents activated by low and high ATP concentrations in more detail. Stimulation with a low ATP concentration (10 μM) induced a small current that declined in the presence of ATP (figure 3.17). The current kinetics are similar to the results obtained before (figure 3.4). However, when the cells where subsequently stimulated with a high ATP concentration (1 mM), the resulting current increased slowly in a biphasic manner. The current maximum was only reached by prolonged stimulation (12 – 85 s) (figure 3.17). The current did not decrease during ATP stimulation and declined only after the ATP stimulation ended. Interestingly, the activation kinetics changed after the initial stimulation with 1 mM ATP. When a cell showed the maximal inducible current once, all
Figure 3.12: Low ATP concentrations activate P2X₄ receptors. (A-D) Modulation of the ATP-induced inward current, exemplary whole-cell voltage-clamp traces, \( V_{\text{hold}} = -80 \text{ mV} \), 10 \( \mu \text{M} \) ATP. (A) Suramin did not inhibit the current activated by a low ATP concentration. (B and C) The ATP-induced current is strongly inhibited by \( \text{Cu}^{2+} \) (100 \( \mu \text{M} \), C) as well as by acidic conditions (pH 6.3, B). (D) Ivermectin (3 \( \mu \text{M} \)) potentiates the ATP-induced current amplitude. (E) Quantification of current amplitudes shown in (A–D). Data are mean ± s.e.m., the number of cells tested is indicated above individual bars, paired Student’s t-Test was used to test for significance: \( p = 0.01 \) (copper), \( p = 6.6 \times 10^{-6} \) (pH 6.3) and \( p = 0.02 \) (ivermectin).
Figure 3.13: Suramin blocks ATP-induced currents in Sertoli cells. (A) Example phase contrast image of a cultured Sertoli cell targeted by the patch pipette. The cell was manually encircled for better visibility. (B) Block of the ATP-induced inward current, exemplar whole-cell voltage-clamp traces. Suramin inhibited the current activated by a low ATP concentration. Higher suramin doses increase the blocking effect, \( V_{\text{hold}} = -80 \text{mV}, 10 \mu\text{M} \text{ ATP} \).

Figure 3.14: Establishing post-transcriptional gene silencing: (A) Successful gene silencing is confirmed by quantitative RT-PCR. Data are normalized to non-transfected cultured spermatogonia and are presented as mean ± s.e.m. Statistical significance was tested with ANOVA / Tukey. While transfection with non-target siRNA (neg.ctr.) has no effect (0.87 ± 0.2), both targeting siRNAs significantly reduce the amount of P2X4 mRNA (RNAi1: \( p = 2.8 \times 10^{-5} \) vs. non-transfected, \( p = 0.001 \) vs. neg.ctr.; RNAi2: \( p = 6.4 \times 10^{-6} \) vs. non-transfected, \( p = 0.002 \) vs. neg.ctr.). (B) Co-transfection with FITC-labelled BLOCK-iTTM fluorescent oligos allowed identification of co-transfected cells by their fluorescence.
Figure 3.15: Post-transcriptional gene silencing confirms the crucial role of P2X₄ receptors in spermatogonial ATP sensing. (A) Original whole-cell voltage-clamp traces, V_hold = -80 mV, 300 µM ATP. Knockdown of P2X₄ receptor expression reduced the ATP-induced current. (B) Quantification of data shown in (A). Data are mean ± s.e.m., the number of cells tested is indicated above individual bars. Significance was tested with ANOVA / Tukey. Knockdown of P2X₄ receptor expression significantly reduced the ATP-induced current (P2X₄ RNAi1: p = 0.02 vs. no transfection, p = 0.02 vs. neg.ctr; P2X₄ RNAi2: p = 0.002 vs. no transfection, p = 0.002 vs. neg.ctr.). P2X₂ receptor knockdown has no effect on the ATP-induced current.
Figure 3.16: Post-transcriptional gene silencing confirms effect of siP2X2 in Sertoli cells. (A and B) Example image of a cultured Sertoli cell targeted by the patch pipette (overlay of the phase contrast image and the FITC fluorescence image). A non-transfected cell shows no fluorescent staining (A) while a transfected cell displays a green fluorescence (B). The cells were manually encircled for better visibility. (C) Exemplary whole-cell voltage-clamp traces of transfected (green) and non-transfected (black) cells. Knockdown of P2X2 decreased the current activated by ATP, $V_{\text{hold}} = -80 \text{ mV}$, 300 μM ATP. (D) Quantification of data shown in (C). Data are mean ± s.e.m., the number of cells tested is indicated above individual bars. Significance was tested with Student’s t-Test ($p = 0.03$). Knockdown of P2X2 receptor expression significantly reduced the ATP-induced current.
Figure 3.17: High ATP concentrations activate additional low sensitivity ATP-receptors. (A) Original whole-cell voltage-clamp trace showing representative currents induced by low (10 μM) and high (1 mM) ATP concentrations. While the current induced by 10 μM ATP desensitizes during the application (1 s) the current induced by 1 mM ATP does not desensitize, but increases with prolonged stimulation (20 s; 1 mM) showing a biphasic current activation kinetic. A second stimulation after a short interval induced a current with a monophasic activation kinetic.

The following stimulations induced a current with a similar maximal amplitude. However, this maximal amplitude was reached quicker and with a monophasic activation kinetic (figure 3.17). Because of these differences, cells were always stimulated with ATP until they displayed the maximal current. We then used responses induced by subsequent stimulations to characterize the current.

Next, we performed repetitive voltage ramps recordings which allows to record the I-V relationships before, during and after ATP stimulation. The leak currents measured before ATP stimulation were averaged and subtracted. The current induced by a low ATP concentration (100 μM ATP) showed inward rectification (3.18; A). This confirms the results we obtained before (figure 3.5). However, the I-V relationships recorded at maximum activation during stimulation with a high ATP concentration (1 mM) revealed an almost linear current with very little rectification (figure 3.18; C). Plotting the current amplitudes measured at -80 mV and +80 mV over time, revealed the same current kinetics we observed before at a constant holding potential (figure 3.18 B and D and figure 3.17).

The detailed analysis of the two current types revealed significant differences in rectification, maximal current, rise time and desensitization rate (figure 3.19). The current activated by a high ATP concentration showed no rectification, no desensitization, reached a larger maximal amplitude and rose much slower than the current induced by low ATP concentrations. These fundamental differences suggest that prolonged stimulation with high ATP concentrations activates other receptors than P2X4, presumable P2X7 recep-
Figure 3.18: Comparison of the ATP-induced I-V relationships: (A and C) I-V relationship of the maximal conductance activated by 100 μM ATP (A) and 1 mM ATP (C). Shown is the mean, the s.e.m. is indicated in gray. 100 μM ATP: n = 16, 1 mM ATP: n = 7. Curves were elicited by voltage ramps as indicated in the inset in (A), ramps were performed every 500 ms. An average control current (leak) recorded before ATP stimulation was subtracted. (B and D) Representative ATP-induced current density plotted over time. Each dot represents the current at -80 mV (white dots) or +80 mV (gray dots) obtained from individual voltage ramps as in (A and C).
Figure 3.19: Quantitative analysis of the I-V relationships revealed distinct current properties: Analysis of the data shown in (figure 3.18). (A) Analysis of the current rectification (current amplitude at -80 mV vs. +80 mV) revealed a significant difference between the currents induced by 100 μM and 1 mM ATP (unpaired Student’s t-Test, p = 2.9 x10^{-7}). Further analysis revealed significant differences in the maximal ATP-induced current density (B, unpaired Student’s t-Test, p = 1.9 x10^{-8} at -80 mV, p = 7.8 x10^{-7} at +80 mV), the time to peak amplitude (C, unpaired Student’s t-Test, p = 2.3 x10^{-8} at -80 mV, p = 0.0001 at +80 mV) and the desensitization rate (D, unpaired Student’s t-Test, p = 3.6 x10^{-6} at -80 mV, p = 6.6 x10^{-9} at +80 mV).
tors. However, at this point, we cannot fully exclude a pore dilation of P2X₄ receptors, although there is evidence that they do not show pore dilation under physiological conditions (Jindrichova et al., 2015). To clarify the identity of the ATP-sensitive receptors, we performed experiments combining pharmacology, RNAi and electrophysiology.

Hallmarks of P2X₇ receptors are their increased sensitivity for BzATP, an artificial ATP analogue, compared to ATP. In addition P2X₇ shows increased current amplitude after prolonged or repetitive stimulation (Surprenant et al., 1996). Thus, if P2X₇ receptors are functionally expressed in spermatogonia, we should be able to induce large, non-desensitizing currents by repetitively stimulating with BzATP. Since P2X₄ receptors are also sensitive to BzATP, we first applied a P2X₄ receptor saturating ATP concentration (300 μM) repetitively. As expected, a P2X₄-like current was induced by the first stimulations and the current amplitude quickly decreased with repeated stimulation. As this ATP concentration is below the activation threshold of P2X₇ receptors, we did not observe a non-desensitizing current during this stimulation. However, subsequent stimulation with BzATP (300 μM) elicited a larger current that increased with repetitive stimulation (figure 3.20) as it is typical for P2X₇ receptor-mediated currents. Knockdown of P2X₄ receptor expression significantly reduced the declining current activated by ATP, while the increasing current activated by BzATP remained unchanged (figure 3.20). This confirms our hypothesis that spermatogonia express (at least) two different ATP receptors and that the large, non-desensitizing currents are not mediated by P2X₄ receptors.

We then analyzed the effect of the P2X₇ receptor specific inhibitor A438079 (Nelson et al., 2006; Donnelly-Roberts and Jarvis, 2007). The desensitizing current induced by ATP (300 μM) was not influenced by the blocker. However, the non-desensitizing current elicited by BzATP (300 μM) was almost completely inhibited (figure 3.21).

We also performed RNA interference experiments to knockdown P2X₇ receptor expression. Therefore, we stimulated naive spermatogonia with a high ATP concentration (1 mM) in patch-clamp experiments. ATP elicited a slowly increasing current that saturated after prolonged stimulation (figure 3.22 A). Interestingly, in ~ 55% of the cells we observed a fast developing small current (first maximum) immediately at the beginning of the stimulation that declined partly within seconds. The I-V relationship of this current showed inward rectification and the current was not influenced by the knockdown of P2X₇ receptor expression, suggesting the activation of P2X₄ receptors (figure 3.22 A and B). The remaining current then increased again very slowly to reach a second, much larger maximal amplitude. The I-V relationship of this second current maximum was
Figure 3.20: P2X₇ receptors are crucial for the detection of high ATP concentrations in spermatogonia. (A) Representative whole-cell voltage-clamp recording showing the currents induced by repetitive ATP and BzATP stimulation (stimulation time 1 s, ISI: 3 s). Spermatogonia were either untreated (black) or transfected with either targeting P2X₄ siRNA (green) or non-target siRNA (neg.ctr., gray). Repeated stimulation with 300 μM ATP induced current desensitization. This current is diminished by knockdown of P2X₄ receptor expression. Subsequent stimulation with 300 μM BzATP (interval 15 s) induces a larger current that increases with repeated BzATP stimulation. This current is not influenced by knockdown of P2X₄ receptor expression. (B) Quantification of data shown in (A). Data are mean ± s.e.m.. Number of cells tested: control n = 33 to 13 (black dots), P2X₄ RNAi n = 16 to 23 (green), neg.ctr. RNAi n = 16 to 23 (gray). Significance was tested with ANOVA / Tukey. Knockdown of P2X₄ receptor expression significantly reduced the ATP-induced current during the first 5 stimulations, p < 0.05.
Figure 3.21: Representative whole-cell voltage-clamp recording revealing the effect of the P2X7 receptor specific inhibitor A438079. (A and B) While the ATP-induced current is not influenced by the blocker (A, control condition black, blocker A438079 10 μM, gray), the maximal BzATP-induced current is strongly diminished (B). (C) Quantification of data shown in (A and B). Data are mean ± s.e.m. and were normalized to their control currents, respectively. The number of cells tested is indicated above individual bars. Significance was tested using the paired Student’s t-Test. A438079 significantly inhibits the maximal BzATP-induced current, p = 0.003.
almost linear without rectification. This current was significantly reduced by the down-regulation of P2X$_7$ receptor expression confirming our hypothesis that this current is mediated by P2X$_7$ receptors. Quantitative PCR confirmed the effective down-regulation of P2X$_7$ receptor mRNA by P2X$_7$-targeting siRNA transfection, while non-targeting control siRNA transfection had no effect (figure 3.22 F).
Figure 3.22: Effects of P2X₇ post-transcriptional gene silencing is limited to the non-desensitizing conductance:
Figure 3.22: Effects of P2X$_7$ post-transcriptional gene silencing is limited to the non-desensitizing conductance: (A) Representative ATP-induced current density plotted over time. Each dot represents the current at -80 mV obtained from individual leak corrected voltage ramps as in (B) and (C). Spermatogonia were either transfected with targeting P2X$_7$ siRNA (green) or with non-targeting control siRNA (neg.ctr., black). The previously unstimulated cells were challenged with 1 mM ATP and responded with immediate current increase that quickly reached a first maximum (first I$_{\text{max}}$, denoted by the cross). This current desensitized to a plateau level followed by a long and slow current increase to a much larger second maximum (second I$_{\text{max}}$, denoted by the asterisk). Only the second I$_{\text{max}}$ is diminished by the knockdown of P2X$_7$ receptor expression. (B) and (C) exemplary I-V relationships of the first and the second maximal current induced by 1 mM ATP. Curves were elicited by voltage ramps spanning -100 to +100 mV in 250 ms, performed every second. The time point of the individual ramp recording is indicated in (A, first I$_{\text{max}}$: cross, second I$_{\text{max}}$: asterisk). (D and E) Quantification of the data shown in (A). Data are mean ± s.e.m., n is the number of cells tested and is indicated above individual bars. Significance was tested and with ANOVA / Tukey. Knockdown of P2X$_7$ receptor expression did not influence the first current maximum (D), while the second current maximum was significantly reduced (P2X$_7$ RNAi: p = 0.02 vs. no transfection, p = 0.03 vs. neg.ctr.). (F) Quantitative PCR shows the successful silencing of the P2X$_7$ receptor encoding gene by transfection with targeting P2X$_7$ siRNA. Data are normalized to non-transfected cultured spermatogonia and are presented as mean ± s.e.m. Statistical significance was tested with ANOVA / Tukey. While transfection with non-target siRNA (negative control, neg.ctr.) has no effect (0.97 ± 0.16), the targeting siRNA significantly reduces the amount of P2X$_7$ mRNA (0.39 ± 0.05, p = 6.6 × 10$^{-5}$ vs. non-transfected, p = 0.0003 vs. neg.ctr.).

3.1.6 Spermatogonia and Sertoli Cells Show Typical ATP-induced Currents in situ.

Having characterized the functional P2X receptor expression in easily accessible dissociated spermatogonia, we aimed to extend our knowledge to spermatogonia in situ. Therefore, we established an acute slice preparation of seminiferous tubules from P7 mice. Figure 3.23 shows an overview of several seminiferous tubules embedded in agarose within a 200 µm thick slice. While some tubules lie horizontally within the slice, some rise perpendicular to the surface and thus cells within the tubule can be accessed by a patch pipette. The application pencil allows stimulation of the whole targeted tubule. As cells in the tubule are not marked, we measured spermatogonia as well as Sertoli cells (figure 3.23 B and D). During the patch-clamp experiment the targeted cell was filled with a fluorescent dye via the patch pipette allowing the reconstruction of the
cell’s morphology. We previously described the functional expression of P2X$_2$ receptors in cultured Sertoli cells Veitinger et al. (2011). When stimulating large cells – presumably Sertoli cells – in this slice preparation with low and high ATP concentrations we observed currents with kinetics typical for P2X$_2$ receptors (figure 3.23 C). However, applying low ATP concentrations on cells with a morphology typical for spermatogonia, that is small and round, induced a current similar to what we observed in the cultured spermatogonia. The current rose relatively fast and declined in the presence of ATP (figure 3.23 E). High ATP concentrations activated a slowly increasing current that only declined after the stimulation (figure 3.23 E).
Figure 3.23: Spermatogonia and Sertoli cells show typical ATP-induced currents in situ.
Figure 3.23: Spermatogonia and Sertoli cells show typical ATP-induced currents in situ. (A, B, D) Representative merged DIC and fluorescence images of the seminiferous tubules slice preparation. (A) Overview of the seminiferous tubules slice preparation used to investigate the P2X receptor expression in situ. Several seminiferous tubules are embedded in the agarose and lie longitudinal or perpendicular to the surface. The tubule in the middle was cut in cross-section and could be targeted by the patch pipette. Filling the patched cell with a fluorescent dye (Alexa 488, here shown in red) via the patch pipette allows morphological identification of the cell type. On the right the application pencil used for perfusion is visible. (B) Magnification of the marked area in (A). The patched cell has a morphology typical for large Sertoli cells. (C) Original whole-cell patch-clamp recordings from morphologically identified Sertoli cells. Low (upper trace) as well as high (lower trace) ATP concentrations induced a non-desensitizing inward current at a holding potential of −40 mV. (D) Exemplary micrograph of a cross-section tubule where a spermatogonium has been targeted by the patch pipette. The fluorescent marker, shown in green, reveals a small round cell. (E) Original whole-cell patch-clamp recordings from morphologically identified spermatogonia. A low ATP concentration induced a current that desensitized in the presence of ATP (upper trace), while a high ATP concentration induced a non-desensitizing current (lower trace), \( V_{\text{hold}} = -40 \text{mV} \).

Comparing the two current types revealed significant differences between the maximal current amplitude, the desensitization rate as well as the maximal amplitude of a second subsequent ATP stimulation (figure 3.24). The current induced by a low ATP concentration had a smaller maximal amplitude and a higher desensitization rate. This desensitization was induced by constant (albeit short) stimulation as well as by repetitive stimulation.

We observed the same differences in the cultured spermatogonia suggesting the same P2X receptor expression in the acute tissue slice. To confirm this we also investigated the effect of pharmacological tools on morphologically identified Sertoli cells and spermatogonia (figure 3.25). Suramin effectively inhibited the current induced by 10μM ATP in Sertoli cells, but not in spermatogonia (figure 3.25 C) concurring with P2X2 receptor expression in Sertoli cells and P2X4 receptor expression in spermatogonia. The P2X7 receptor blocker A438079 had no effect on the current induced by 1 mM ATP in Sertoli cells confirming our previous finding that these cells do not functionally express P2X7 receptors (figure 3.25 G; Veitinger et al. 2011). However, A438079 significantly reduced the current induced by 1 mM ATP in spermatogonia (figure 3.25 I and H), showing that high ATP concentrations activate P2X7 receptors in these cells.
Figure 3.24: Quantitative comparison of spermatogonia and Sertoli cells *in situ* current kinetics: Analysis of the currents induced by 10 μM ATP (low conc.) and 1 mM ATP (high conc.) in spermatogonia. Data are mean ± s.e.m., number of cells tested is indicated above individual bars. There are significant differences in the maximal current amplitude (A, p = 0.01, paired Student’s t-Test) and the desensitization rate (B, p = 0.007, Student’s t-Test). When spermatogonia were stimulated twice (C), the current amplitude induced by the second stimulation with 10 μM ATP was significantly smaller than the first one (p = 0.01, paired Student’s t-Test). Repetitive stimulation with 1 mM ATP did not induce a current decrease. The normalized second current amplitude differed significantly from the second current amplitude induced by 10 μM ATP (p = 0.0007).
Figure 3.25: Pharmacological characterization of ATP-induced currents in seminiferous tubules slices.
Figure 3.25: Pharmacological characterization of ATP-induced currents in seminiferous tubules slices: (A, B, D and E) Confocal and DIC micrographs of tubules targeted by the patch pipette. Cells were filled with a fluorescent dye via the pipette. For easier distinction Sertoli cells are shown in red (A and B), while spermatogonia are shown in green (D and E). Note in the upper pictures the cup-like processes of the Sertoli cell that likely envelope round spermatogonia (not stained). In the lower pictures the targeted spermatogonium is connected to a second spermatogonium. (B) Exemplary whole-cell voltage-clamp traces showing the effect of suramin on the ATP-induced current in Sertoli cells (upper, red trace) and spermatogonia (lower, green trace). ATP, 10µM. (G and I) Exemplary whole-cell voltage-clamp traces showing the effect of the P2X7-specific inhibitor A438079 on the ATP-induced current in Sertoli cells (G, red trace) and spermatogonia (I, green trace; 1 mM ATP). (D) Quantification of the data shown in (B) and (C). Cell types are color coded. Data are mean ± s.e.m., number of cells tested is indicated above individual bars. Significance was tested with paired Student’s t-Test. Suramin significantly inhibits the current induced by 10µM ATP in Sertoli cells (p = 0.03) but not in spermatogonia. A438079 significantly reduced the current induced by 1 mM ATP in spermatogonia (p = 0.02) but not in Sertoli cells.
3.2 Excursus 1: Deletion of GAR22$\beta$ gene impairs spermatogenesis and spermatozoa motility

As male reproduction was in the center of our studies we had the opportunity to contribute valuable data to a project spearheaded by our cooperation partners from the Institute of Biomedical Engineering, Dept. of Cell Biology, Uniklinik RWTH Aachen. This project aimed to reveal the function of the Gas2-related protein on chromosome 22 (GAR22$\beta$), a poorly characterized protein that interacts with microtubules and actin.

To investigate the function of GAR22$\beta$, our collaboration partners generated a GAR22$\beta$ knockout mouse. The exons 3-6 and most of exon 7 of the GAR22$\beta$ gene were replaced with neomycin and LacZ cassettes, leading to the complete loss of GAR22$\beta$ (and its splicing variant GAR22$\alpha$) expression (figure 3.26 A). It turned out that the male GAR22$\beta$ knockout mice showed a strongly reduced reproductive potential, indicating that GAR22$\beta$ is important for sperm function, spermatogenesis, or both.

We investigated the expression pattern of GAR22$\beta$ in the tubules seminiferous of adult and juvenile mice using histochemistry. Due to the expression of LacZ, the (former) location of GAR22$\beta$ could be determined by staining in the knockout mice. Furthermore, we used an antibody against GAR22$\beta$ to investigate the location in wildtype mice. We found that GAR22$\beta$ is robustly expressed in germ cells located in the adluminal part of the tubules. However, GAR22$\beta$ expression was not visible in the tubules seminiferous of juvenile P7 mice (figure 3.26 C).

In juvenile testis, seminiferous tubules consist exclusively of immature highly proliferative Sertoli cells and type A spermatogonia (Bellve, 1977; Veitinger et al., 2011), suggesting that substantial GAR22$\beta$ expression coincides with germ cells entering meiotic and or postmeiotic stages of the seminiferous cycle.
Figure 3.26: GAR22β is expressed in seminiferous tubules of adult but not juvenile mice. (A) Schematic showing the exon-intron structure of the GAR22β gene. The part highlighted in blue is corresponding to the open reading frame. (B) RT-PCR analysis of GAR22α and GAR22β expression in cultured Sertoli cells isolated from wild type or GAR22β KO testes. GAPDH served as loading control. (C) Expression of GAR22β in juvenile and adult seminiferous tubules. Cryosections of wild type (WT), heterozygote (HET) and GAR22β−/− (KO) testes were stained with X-Gal solution. GAR22β was primarily expressed in adluminal regions of seminiferous tubules. Dotted boxes indicate the area enlarged in the inset (right corner; B and C). Scale bars main images: 100 µm; / inset: 50 µm.

In adult testis, we found substantial GAR22β co-localization with the actin cytoskeleton. In wild type seminiferous tubules antibody staining against GAR22β showed that the spatial overlap is most pronounced at the adluminal Sertoli cell junctions / apical ectoplasmic specialization (figure 3.27). No such signals were observed in seminiferous tubules from GAR22β knockout mice.
Figure 3.27: GAR22β colocalizes with actin in seminiferous tubules: Cryosections of wild type testes were stained with fluorescent phalloidin (f-actin marker), GAR22β antibodies and DAPI (nucleus staining) and then analyzed by confocal microscopy. Actin was distributed throughout the seminiferous tubules where it collocates in many regions with GAR22β. Testicular cryosections of knockout mice showed only nonspecific anti-GAR22β signals. Interestingly, also the actin fibers stained by phalloidin appeared less structured. The small inset depicts the nuclei staining (DAPI). In the merged image, actin is shown in red, GAR22β in green. Scale bar: 50 μm.

Notably, the loss of the GAR22β was concomitant with a strong decrease in f-actin expression in seminiferous tubules (figure 3.28). F-actin reduction was most pronounced at the basal membrane and the luminal ES surrounding elongated spermatids (figure 3.28). Together, these results suggest that GAR22β is critically involved in proper spermatid elongation and axoneme development.
Figure 3.28: Deletion of GAR22β causes massive reduction of actin levels in mouse testes. Cryosections of wild type and GAR22β KO testes were stained with fluorescent phalloidin and analyzed by confocal microscopy. Upper panel: Actin was distributed throughout the seminiferous tubules in wild type testes. Both, at the periphery and in the central region of single tubules, a robust actin labeling was detected. Actin was accumulated in the adluminal regions, particularly in areas corresponding to spermatozoa heads (arrows). Lower panel: In GAR22β−/− (KO) testes, by contrast, the adluminal level of actin was to a large extent reduced with little or no actin around spermatozoa heads (arrows). Dotted boxes indicate the area enlarged in the inserts. Green phalloidin and red nuclear staining (DRAQ5). Scale bars main images: 50 μm; / inset: 25 μm.
3.3 Excursus 2: CLARITY for Rapid Clearing and Imaging of Intact Anatomical Structures

During this thesis we also established the CLARITY method (Chung and Deisseroth, 2013) in our laboratory.

CLARITY is a method to transform (entire) biological structures (organs or body parts) into a specimen that can be analyzed by light microscopy. In untreated biological tissue samples, light is scattered by many distributed water based compartments, cell membranes and proteins. Those materials have diverse optical properties. To allow deep light penetration into a specimen it is necessary to reduce these inhomogeneous optical properties (especially the optical density or RI) in the tissue (3.29 A and B). The CLARITY method is designed to analyze the occurrence and position of target proteins within an intact tissue structure. Therefore, all proteins are retained at their original position, while all other light scattering cellular structures are removed.

figure 3.29 C and D a P1 mouse head before and after clearing using the CLARITY method. While the uncleared mouse head still scatters most of the light at the very surface, the cleared head allows light to pass through its whole volume.

figure 3.29 E provides an overview of the method. CLARITY aims to remove lipids from the plasma membrane and to substitute all water based compartments by a solution matching the RI of most proteins. However, lipids provide an important structural component of fixated tissue samples. To maintain structural integrity, the fixated proteins were cross-linked to acrylamide monomers. Those can, in turn, polymerize to a macroscopic supporting structure. Electrophoretic tissue clearing provides a fast way to remove the lipids from the specimen.
Figure 3.29: CLARITY for rapid clearing of intact anatomical structures.
Figure 3.29: CLARITY for rapid clearing of intact anatomical structures.

(A and B) Schematic illustration showing benefits of cleared tissue (B) compared to uncleared tissue (A) in light microscopy. (A) Light is scattered by the uncleared tissue due to many layers of cell membranes which individually differ in their RI compared to the extracellular or cytosolic RI. Originally precisely defined structures appear diffuse during standard imaging methods. Maximum penetration depth with high end multi photon microscopy is limited to about 200\(\mu\)m. (B) Cleared tissue allows the light to travel through its focal point and back to the Objective without diffusion by layers of varying RI. Defined structures appear clearly visible during imaging. The maximum penetration depth increases dramatically depending on clearing effectiveness. (C and D) Head of a P1 Mouse before and after clearing. The specimen was processed as a hole including bone and cartilage. (C) The untreated specimen appears dense and the light is reflected or scattered at the surface of the specimen. (D) The cleared specimen conducts light that has been reflected by the underlying text, indicating limited light scattering across the whole specimen. (E) Illustration of tissue embedding and lipid removal for CLARITY. (Left) Tissue is perfused or immersion loaded with HM (v). PFA (red) links free floating monomers to proteins. (Middle) Polymerization of the hydrogel (37°C; O\(_2\)-free conditions), it forms a matrix which stabilizes the cross-linked proteins (dark blue). (Right) Electrophoresis together with SDS removes the lipids from the specimen reducing variations in RI.

To enable accurate light microscopic imaging of cleared specimen, optimized optics and advanced microscopic approaches were used. The HC FLUOTAR L 25x/1.00 IMM (n\(_e\)= 1.457) objective is optimized for high RI solutions and cleared tissues and has a 6 mm working distance. The working distance is an important feature for imaging CLARITY specimens. As described in chapter (figure 3.30 A), not only the penetration depth of the light but also the macroscopic structure of the specimen can easily become an obstacle.

For better z-resolution two photon imaging was used. To test the established method, we used an OMP-GFP P1 mouse, in which all mature neurons of the olfactory system express the fluorescent protein GFP. After clearing the whole head, we acquired 2600 images with a z-step size of 880 nm to travel 2.1 mm deep into the tissue. The z-stack was virtually reconstructed to a 3D object using Imaris. Even in deeper regions the image quality is sufficient to identify sub-cellular structures (figure 3.30 D).
Figure 3.30: Imaging a CLARITY specimen
Figure 3.30: Imaging a CLARITY specimen: (A) Experimental set-up to image cleared specimen. The HC FLUOTAR L 25x/1.00 IMM (n_e = 1.457) objective is used to image the olfactory system in a cleared P7 OMP-GFP mouse head. Note that the light can travel through the whole specimen. (B) Schematic view of (A), all structures containing OMP are marked in green. The position of the eye is depicted in light pink. (MOB: Main olfactory bulb, CP: cribriform plate, MOE: main olfactory epithelium) (C and D) virtual 3D reconstruction of 2,600 z-planes of a cleared P1 OMP-GFP mouse head. (C) Frontal view of the 3D reconstructed GFP fluorescence. At the top the two olfactory bulbs are visible (MOB). Nerve fibers pass through the cribriform plate (CP) to the left main olfactory epithelium (MOE). (D, left) sagittal view of the 3D reconstruction. The left MOB and MOE are connected by numerous nerve fibers. (D, right) original images from the z-stack connected by lines to the position in the 3D reconstruction. Note that the images allow to identify subcellular structures through the whole z-stack. In the image of the MOB single glomeruli can be identified. Here, the axons of the olfactory sensory neurons connect to the dendrites of the first order brain neurons (mitral cells). In the image of the CP region nerve fibers and axon bundles can be resolved. The image of the MOE even depicts single olfactory sensory neurons, which are aligned around the lumen of the nose. Note that even the small structures of the dendrites and the knob region can be resolved.
Chapter 4

Discussion

Understanding the reproductive functionality of the male body is important in many aspects. Reproduction itself is essential for species survival. In male reproduction, spermatogenesis plays a central role. So far, however, knowledge about the physiological processes taking place during spermatogenesis is sparse. Therefore, more information is needed to provide knowledge about the physiological basics in this system. The gained information is also important for our society as, depending on the individual way of life, human reproductive capability can be either desirable or undesirable. Thus, deeper knowledge about spermatogenesis can offer ways to improve the quality of life in a modern society.

In this study we investigated the functional expression of ion channels in spermatogonia. Ion channels are widely used in cellular communication. We started our work with the hypothesis that ATP is an important extracellular messenger during spermatogenesis. We and others could show a role for ATP in the somatic cells in the seminiferous tubules, the Sertoli cells (Filippini et al., 1994; Rossato et al., 2001; Veitinger et al., 2011; Burnstock, 2014). Here, we focused on the stem cells in the seminiferous tubules, the spermatogonia. We present the first physiological evidence that multiple ATP-sensitive P2X receptors are functionally expressed in spermatogonia.
4.1 Electrophysiological Experiments with Extracellular ATP in Prepubescent Spermatogonia

4.1.1 Targeting Spermatogonia

The developing germ cells in the adult seminiferous tubule are diverse. They undergo dramatic morphological changes and before, during and after meiosis Kerr et al. (2006).

During spermatogenesis there is a huge variety of different germ cell stages. A histological study investigated the expression of ATP sensitive ionotropic P2X receptors in the testes of adult rats. They described that the expression pattern of P2 receptors variates over the seminiferous cycle as well as during germ cell maturation (Glass et al., 2001). For our study it was therefore crucial to isolate germ cells in a specific state in order to avoid an inhomogeneous ion channel expression pattern.

We focused on spermatogonia as the primordial subset of germ cells. Using juvenile reproductive tissue for our culture system offered the advantage to avoid the diverse germ cell composition of adult seminiferous tubules. Juvenile seminiferous tubules exclusively comprise spermatogonia (Bellve, 1977; Kluin et al., 1984; Kerr et al., 2006; Hermo et al., 2010) (figure 1.3) and Sertoli cells. Using this approach we succeeded to culture spermatogonia with high purity. Which subtype of spermatogonia were included in our culture is very difficult to determine. Morphologically the definition of the germ cell stage, with the exception of undifferentiated type A spermatogonia, is coupled to the seminiferous cycle. Obviously, this categorization cannot be applied to our dissociated spermatogonia in a culture dish. Some immunohistological markers have been implicated to categorize spermatogonia according to intrinsic expression patterns (Kolasa et al., 2012). We used the antibody against DAZL because binding of this antibody identifies the cells as premeiotic germ cells (De Reijo et al., 1996; Ruggiu et al., 1997; Niederberger et al., 1997; de Rooij and Russell, 1997). As expected, in adult testis slices only cells located close to the basement membrane and in juvenile testis slices almost all cells were stained, confirming the described antibody targets. We then established the DAZL staining in our primary cell culture from juvenile mouse testis. The results confirm the purity of our culture system. This supports that our experimental design is suitable to investigate a defined subset germ cells, namely spermatogonia (Veitinger, 2009).
4.1.2 Identifying ATP-sensitive Channels in Spermatogonia

Performing patch-clamp experiments with single spermatogonia, we discovered a fast activating current induced by ATP in about 60% to 80% of the targeted cells (see figure 3.1).

Stimulation with 30\(\mu\)M ATP for 300 ms induced stable responses. However, it was not sufficient to induce the maximal current. This is likely due to the fact that not all ATP-sensitive receptors were activated at the same time. Prolonging the stimulation to 1 s lead to a saturated response with minimal desensitization during the stimulation, showing that this stimulation is sufficient to activate all receptors. Even more prolonged stimulation induced a current decline during stimulation, indicating receptor desensitization. In accordance with these results we decided to use 1 s stimulations in most of our experiments.

Next, we investigated the influence of stimulus repetition (using 100\(\mu\)M ATP) on the current amplitude. Decreasing the ISI revealed that the current amplitude declines during repeated stimulation with short intervals. However, an ISI of 60 s is sufficient to minimize these desensitization effects. Therefore, a stimulation interval of at least 60 s was used in all following experiments.

The I-V relationship of the instantaneous ATP-induced current displayed strong inward rectification (figure 3.5 and figure 3.8).

The reversal potential was around 0 mV. The delayed outward current that occurred at holding potentials more positive than -80 mV in a subset of ATP-sensitive cells (3.7), will be discussed later (4.1.4). The instantaneous current showed a dose-dependency with an \(EC_{50}\) of 13.5 \(\mu\)M. The additional current activated by ATP concentrations higher than 300 \(\mu\)M will be discussed later (see: 4.1.5). Taken together, we observed an ATP-induced current with moderate desensitization during prolonged stimulation (>1 s), inward rectification and an \(EC_{50}\) of 13.5 \(\mu\)M. These features are characteristics of currents mediated by ATP-sensitive P2X\(_2\), and/or P2X\(_4\) receptors (Brake et al., 1994; Eickhorst, 2002; Khakh et al., 1999; Jones et al., 2000; Casas-Pruneda et al., 2009; Bo et al., 1995; Evans et al., 1996; Townsend-Nicholson et al., 1999; Clyne et al., 2003; Fujiwara and Kubo, 2004; Fountain and North, 2006; Coddou et al., 2011). In a different set of experiments we used voltage ramp recordings to investigate the I-V relationship (3.18; 3.19). The I-V relationship obtained from these ramp recordings also showed rectification, however, it was less pronounced. This can be explained by the fact that during voltage ramp experiments a short ATP stimulation was sufficient since the different holding potentials were all measured within 300 ms. Thus, possible rundown effects (Fountain and North,
2006; Zemkova et al., 2014a) as well as desensitization are minimized.

Using RT-PCR we found three different P2X receptors (P2X\textsubscript{2}, P2X\textsubscript{4} and P2X\textsubscript{7}) in testicular tissue from our primary cell culture (figure 3.10). Other groups identified more and /or other P2X receptors in testicular tissue (Bo et al., 1995; Collo et al., 1996; Glass et al., 2001). However, they investigated testicular tissue from adult rats which is likely the reason for the discrepancies. In adult rat testis tissue P2X\textsubscript{4} and P2X\textsubscript{6} receptors were found using RT-PCR (Bo et al., 1995; Collo et al., 1996). Glass et al. (2001) found P2X\textsubscript{1}, P2X\textsubscript{2}, P2X\textsubscript{3}, P2X\textsubscript{5} and P2X\textsubscript{7} using immunohistochemistry. Some of the published data were conflicting. In contrast to this studies we used juvenile mouse tissue, this may explain the difference to other findings. The harvesting of the cells for RNA isolation was performed using repetitive pipetting onto the cells to extract the RNA predominantly from spermatogonia. However, we cannot exclude that also Sertoli cells were part of our preparation. Our group previously showed that P2X\textsubscript{2} receptors are functionally expressed in Sertoli cells (Veitinger et al., 2011). Hence, the P2X\textsubscript{2} signal could originate only from Sertoli cells or from Sertoli cells and spermatogonia.

We also used Western blot analysis and immunohistochemistry to analyze testis tissue. We found a broad distribution for both, P2X\textsubscript{4} and P2X\textsubscript{7} within the juvenile seminiferous tubules. Due to the proximity of the cellular staining it was not possible to differentiate between Sertoli cells and spermatogonia.

4.1.3 Selective Pharmacological Ion Channel Inhibitors and Modulators

The analysis of the kinetic features of the ATP-induced currents in combination with the molecular / immunohistological analysis pointed to an involvement of P2X\textsubscript{2} and / or P2X\textsubscript{4} receptors in the ATP-induced response in spermatogonia. To further differentiate between the two receptors we used selective pharmacological ion channel inhibitors and modulators. First, we used suramin which is a rather unselective P2X receptor antagonist (North, 2002). However, for our purpose it is very useful as it is a potent P2X\textsubscript{2} receptor inhibitor (Brake et al., 1994; Evans et al., 1995), but does not or only slightly inhibit rat or mouse P2X\textsubscript{4} receptors (Buell et al., 1996; Soto et al., 1996; Jones et al., 2000). Townsend-Nicholson et al. (1999) even reported a suramin-induced potentiation of mP2X\textsubscript{4}-mediated responses. In our experiments suramin (10\mu M or 100\mu M) had no effect on the ATP-induced response in spermatogonia (figure 3.12). As the lack of inhibition might be due to an ineffective substance, we verified the suramin potency
by investigating Sertoli cells present in the same cell culture. We showed before that these cells express P2X$_2$ receptors (Veitinger et al., 2011) and, as expected, were able to showed a competitive inhibition of the ATP-induced current in these cells with suramin (figure 3.13). These experiments indicate that P2X$_2$ receptors are not functionally expressed in spermatogonia.

We further investigated the effects of acetic pH and Cu$^{2+}$. Both are reported to potentiate P2X$_2$-mediated currents (King et al., 1996; Xiong et al., 1999), while P2X$_4$-receptors are inhibited (Stoop et al., 1997; Acuna-Castillo et al., 2000; Coddou et al., 2003, 2007). Our observation that the ATP-induced current in spermatogonia is strongly inhibited by acidic pH and Cu$^{2+}$ argues again for the functional expression of P2X$_4$- but not P2X$_2$-receptors in these cells.

Additionally to pharmacological inhibitors we also used the P2X$_4$ receptor specific modulator ivermectin (Khakh et al., 1999; Casas-Pruneda et al., 2009), which leads to significant current increase in spermatogonia. This argues again for the activation of P2X$_4$ receptors as P2X$_2$ receptors are not affected by ivermectin (Khakh et al., 1999). Taken together, this pharmacological profile shows the functional expression of P2X$_4$ receptors in spermatogonia. However, we do not find any evidence for the involvement of P2X$_2$ receptors. Therefore, the evidence for P2X$_2$ receptor expression found in the PCR experiments is likely to originate from Sertoli cells. We also found evidence for the expression of P2X$_7$ receptors in the PCR analysis. Since the low ATP concentrations (10$\mu$M) used in the experiments so far discussed is too low to activate P2X$_7$ receptors, we performed another set of experiments to investigate a possible functional expression of P2X$_7$ receptors. These results will be discussed later (section 4.1.5).

4.1.4 Calcium-activated Potassium Channels Mediate the Delayed Current.

A subpopulation of ATP sensitive cells showed a delayed current right after the ATP-induced current (section 4.1). This current reversed at -80 mV (figure 3.5; figure 3.6; figure 3.7) and did not develop when the internal potassium was replaced by cesium. Furthermore, it was blocked by extracellular TEA and IBTX and depended on extracellular calcium (figure 3.8).

The reversal potential of -80 mV is close to the calculated equilibrium potential for potassium (-85 mV) in our solutions (a)(e) and indicates an activation of potassium channels. The absence of the current using intracellular cesium solution supports this
hypothesis as many potassium channels do not conduct cesium. The inhibition by TEA also indicates the activation of potassium channels (Yellen, 1984). To our knowledge there are no potassium channels that are directly activated by extracellular ATP. We therefore hypothesized a downstream activation mediated by the calcium influx through the ATP-activated P2X receptors. Using immunohistochemistry and Western blot analysis we found evidence for BKCa channel expression in the juvenile tubules seminiferous tissue (figure 3.11).

To support this finding we performed physiological experiments with a calcium reduced extracellular solution (c). Under this condition the ATP-induced response changed in two ways: first, the instantaneous current (mediated by P2X4-receptors) was increased; second, the delayed current was absent. Both effects were reversible. This indicates that under control conditions ATP opens P2X receptors allowing an influx of extracellular calcium which, in turn, activates a calcium dependent potassium channel.

There are several reports describing an increase of the current mediated by P2X2 receptors under reduced extracellular Ca$^{2+}$ conditions (Nakazawa and Hess, 1993; Evans et al., 1996; Khakh et al., 1999; Ding and Sachs, 1999b,a). This can be explained by a saturable binding site for Ca$^{2+}$ ions within the channel pore. A high Ca$^{2+}$ concentration therefore blocks the channel for permeation by other ions (Ding and Sachs, 1999b,a). For P2X4 receptors this phenomenon is less well characterized. However, a modulation of the current amplitude by extracellular Ca$^{2+}$ has been described (Khakh et al., 1999). Thus, the increased instantaneous current amplitude under Ca$^{2+}$ reduced conditions in our experiment could be caused by a relief of the partial Ca$^{2+}$ ion block under control conditions. Additionally, P2X2, P2X4 and P2X7 receptors have a higher affinity to ATP$^4-$ compared to divalent saturated ATP$^{2-}$ (Yan et al., 2011; Li et al., 2013; Riedel et al., 2007). In a Ca$^{2+}$ reduced extracellular solution more ATP$^4-$ is present. This could shift the dose response curve to the left. A non-saturating stimulus (30 μM ATP) could then, in turn, induce an increased current.

To further verify the identity of the Ca$^{2+}$-depended channels expressed by spermatogonia, we used IBTX which is a selective blocker for BKCa channels (Galvez et al., 1990). It selectively blocked the delayed current component, while the instant current component remained unaltered. While the existence of BKCa-mediated currents has been postulated in rat spermatogonia by kinetic analysis (Hagiwara and Kawa, 1984; Gong et al., 2002), we present the proof that BKCa channels are functionally expressed in mouse spermatogonia and that they are activated downstream of P2X receptor activation by the influx of extracellular ATP. The intracellular Ca$^{2+}$ concentration is usually
strictly controlled and an increase is often spatially limited (Clapham, 2007). Therefore, the downstream activation of BKCa channels suggests that they are located in near proximity of the ATP-sensitive P2X receptors in the plasma membrane.

Moreover, we showed a functional coupling of the two different channels by investigating their effect on the membrane potential (figure 3.9). The cation influx through the ATP-activated P2X receptors induced a membrane depolarization. Interestingly, the downstream activation of the BKCa channels lead to a relatively fast repolarization and even a hyperpolarization. Then, the membrane potential returned to its resting value. In principle, this describes an event resembling a slow action potential in these non-excitable cells. It is tempting to speculate that this bioelectric signal could be involved in proliferation, differentiation and/or apoptosis as it has been implicated for other cell types (Sundelacruz et al., 2009; Franco et al., 2006; Wang, 2004). Additionally to the membrane potential changes the ATP-induced Ca\(^{2+}\) influx can play many different roles in spermatogonia, as Ca\(^{2+}\) is known for its versatile influence as second messenger (Hardingham and Bading, 1999; Stolze et al., 2015). The protein "calcium and integrin binding 1" (CIB1) could be one important player in that Ca\(^{2+}\) cascade. CIB1 was found in round and condensing spermatids. It was shown that Cib1\(^{-/-}\) male mice are sterile due to absence of the haploid phase of spermatogenesis (Yuan et al., 2006).

Taken together, our work provides the basis for future studies to gain more detailed insight into the processes underlying spermatogenesis.

### 4.1.5 High ATP Concentrations Activate Additional Low Sensitivity ATP-Receptors

Using higher ATP concentrations we found dramatic changes in the ATP-induced conductivity (figure 3.17). First, we noticed the biphasic activation kinetic that transformed into a monophasic kinetic after repetitive or prolonged stimulation. Recording repeated current-voltage relationships and plotting current amplitudes over time allows precise analysis of several kinetic features at once (rectification, maximum conductance, time to peak, desensitization). The currents induced by 100\(\mu\)M and 1 mM ATP (monophasic kinetic) differed in all these features, indicating that there are two different channel types involved. Since these additional channels are only activated by very high ATP concentrations, they are rather insensitive ATP receptors. The most insensitive P2X receptor known is the P2X7 receptor (North and Barnard, 1997). Intriguingly, these receptors show a biphasic activation kinetic during first stimulation that changes into a
monophasic activation kinetic with repeated stimulation (Nuttle and Dubyak, 1994; Li et al., 2015). Additionally, P2X7 receptor-mediated currents do not display strong rectification (Surprenant et al., 1996; Virginio et al., 1997). Combining these results with the fact that we found P2X7 receptor mRNA in the PCR experiments clearly points to a functional expression of P2X7 receptors in spermatogonia.

We used pharmacology to verify this hypothesis. Indeed, the selective P2X7 receptor inhibitor A438079 (Nelson et al., 2006; Donnelly-Roberts and Jarvis, 2007; Donnelly-Roberts et al., 2009) blocked the currents induced by high ATP concentrations in spermatogonia. However, currents induced by the lower ATP concentration (100μM) were not changed by the blocker, indicating that this ATP concentration is not sufficient to activate P2X7 receptors in spermatogonia. Functionally, P2X7 receptor can play a role in a variety of cellular responses including apoptosis, inflammation and even proliferation (Coutinho-Silva et al., 1999; Adinolfi et al., 2005; Donnelly-Roberts and Jarvis, 2007). Recently, it has been reported, that the inhibition of mitochondrial respiration decreases the germ cell apoptosis (Erkkila et al., 1999, 2003, 2006). The impairment of mitochondria leads to decreased ATP levels in the tissue, thus an involvement of P2X7 receptors in this respiration dependent apoptosis pathway is possible (Burnstock, 2014).

4.1.6 RNAi Knockdown of P2X Receptors in the Testicular Tissue

4.1.6.1 RNAi Knockdown of P2X2

We used RNAi experiments to further validate our results. First, we tested the effect of two different siRNAs against P2X2 receptors on Sertoli cells and spermatogonia. While a significant effect on Sertoli cells was present, no significant alteration of the ATP-induced current in spermatogonia was detected. This finding confirms our previous results Veitinger et al. (2011) as well as our data obtained in the pharmacological differentiation between P2X2 and P2X4 receptors. Furthermore, we used these experiments as proof that RNAi experiments are working in our hands.

4.1.6.2 RNAi Knockdown of P2X4

To verify the functional expression of P2X4 receptors in spermatogonia we aimed to downregulate receptor expression using RNAi. Successful downregulation of the target protein expression should be controlled on different levels. To preclude side effects from the transfection procedure we used non-target siRNA. In our hands the non-target siRNA
did not influence the ATP-induced currents. Great care was also taken regarding the used siRNA concentration as well as the incubation time. We used low concentrations to avoid possible side effects. The incubation time was maximally 24 h to keep a possible upregulation of other P2X receptors at a minimum (Weinhold et al., 2010). To assure the specificity of the target siRNA we used two different siRNAs targeting the same protein. Both siRNAs had the same effect on the ATP-induced currents. Finally, we used quantitative RT-PCR to control the amount of targeted mRNA. Therefore, we are confident that we successfully downregulated the target protein expression using RNAi. This knockdown led to a significant reduction of the ATP-induced currents in spermatogonia (stimulated with ATP <300µM), showing that low ATP concentrations indeed activate P2X4 receptors. This result concurs with our biophysical characterization and the pharmacological profile of the ATP-induced current. The biophysical characterization and the pharmacological profile of the current induced by high ATP concentration (≥ 1 mM) point to an activation of P2X7 receptors. However, we cannot fully exclude a contribution of pore-dilated P2X4 receptors to this enlarged current. Therefore, we designed an experiment combining the molecular manipulation via RNAi with a pharmacological approach. We stimulated the spermatogonia repetitively with an ATP concentration that is saturating for P2X4 receptors, followed by repetitive stimulation with BzATP. The ATP stimulation activated a characteristic P2X4 receptor current that decreased with increasing number of stimulations. This behavior is typical for P2X4 receptors (Fountain and North, 2006; Zemkova et al., 2014b). After almost complete desensitization of the responses typical for P2X4 receptors, we continued stimulation using BzATP (300µM). At this concentration BzATP activates P2X4 and P2X7 receptors (He et al., 2003), the later one displays an even higher sensitivity for BzATP than for ATP (Chessell et al., 1998). BzATP instantly induced a larger current. Instead of desensitization, a current increase over the consecutive stimulus repetition occurred. To investigate if pore-dilated P2X4 receptors contribute to this current, we also used cells in which the P2X4 receptor expression has been downregulated by RNAi. The transfection exclusively altered the current amplitude of the desensitizing current induced by ATP. The residual desensitizing P2X4-like current was significantly smaller but desensitized with the same rate as the P2X4-like current in non-transfected cells (data not shown). This was expected as the downregulation is not complete. The increasing currents induced by repetitive BzATP stimulation were not influenced by the transfection. This shows that there is no contribution of P2X4 receptors to this current. Our data are in accordance with previous findings that P2X4 receptors show pore dilation only in Ca2+-deficient medium.
or in the presence of ivermectin (Khakh et al., 1999; Zemkova et al., 2014a). Our results also indicate that ATP concentrations $\leq 300\mu$M activate only P2X$_4$ receptors, while the influence of the P2X$_7$ receptors at these concentrations seems negligible.

4.1.6.3 RNAi Knockdown of P2X$_7$

We also tested spermatogonia treated with siRNA against P2X$_7$ receptors. Here, we chose to activate the cells with a prolonged 1 mM ATP stimulation. This stimulation is saturating for P2X$_4$ and also effective for P2X$_7$ receptors figure 3.22. Roughly half of the cells showed a characteristic P2X$_4$ receptor-mediated current that desensitized and had an inwardly rectifying I-V relationship. This matches the percentage of ATP-sensitive spermatogonia we measured before using low ATP concentrations figure 3.1. All the cells tested displayed a large, slowly developing, non-desensitizing inward current likely mediated by P2X$_7$ receptors. The I-V relationship of this current showed little rectification. While the desensitizing component at the beginning of the experiment was not influenced by the downregulation of P2X$_7$ receptor expression, a significant reduction of the subsequent large current absolute peak was observed. The experiment reveals that this large current is mediated by P2X$_7$ receptors. It also shows that P2X$_7$ receptors can be manipulated independently from P2X$_4$ conductivities.

Interestingly, we found that all spermatogonia functionally express P2X$_7$ receptors, while the expression of P2X$_4$ receptors is limited to approximately half of the cells. At this point we do not know if P2X$_4$ receptors are expressed by a specific subset of spermatogonia. Further immunohistological characterization might help to differentiate the different types of spermatogonia and which cells express P2X$_4$ receptors.

4.1.7 Spermatogonia and Sertoli Cells Show Typical ATP-induced Currents in situ

The acute tissue slice preparation is a very important step to perform physiological experiments in the male reproductive tissue. We are the first group successfully establishing a patch-clamp experiment in an acute slice preparation in testis tissue. Dissociated cells are better suitable to perform ligand-gated ion channel characterizations due to their easy accessibility for the tested ligands/pharmaceuticals. Our conclusions on the functional P2X receptor expression has been confirmed by our molecular biological as well as our immunohistological data obtained from acute tissue preparations. However, we cannot fully exclude that the cell culture influences the P2X receptor expression levels.
(King, 2006). We therefore chose to repeat key experiments in the acute slice preparation where the cells maintain their original connections, shape and ion channel configuration. Here, we introduced an acute tissue slice preparation form juvenile mice. We performed patch-clamp experiments to confirm our findings from the spermatogonia cell culture. In addition, the acute tissue slice preparation can provide new insides into the intercellular properties of the reproductive tissue. Sanchez-Cardenas et al. (2012) shows synchronized calcium oscillations using calcium imaging within the adult seminiferous tubules. Future experiments can utilize our preparation to address the question if these oscillation already occur in juvenile testis.

We were able to identify the different cell types by fluorescent dye-loading via the patch pipette. Dye filled Sertoli and germ cells were well distinguishable by their shape. We used the same ATP concentrations as before and recorded cell type specific current kinetics in both populations figure 3.23. Sertoli cells show a slow or non-desensitizing ATP-induced (P2X$_2$-like) current, as shown in Veitinger et al. (2011). This current occurred using high and low ATP concentrations (10$\mu$M and 1 mM). Spermatogonia showed a desensitizing ATP-induced (P2X$_4$-like) current when 100$\mu$M of ATP was used and a slowly increasing P2X$_7$-like response, when 1 mM ATP was used.

We confirmed the identity of the mediating receptors by inhibition with suramin (used as P2X$_2$ but not P2X$_4$ receptor inhibitor) and A438079 (P2X$_7$ receptor inhibitor). Suramin exclusively inhibited the ATP-induced current in Sertoli cells but not in spermatogonia (10$\mu$M ATP). The putative P2X$_7$ conductivity in spermatogonia could be successfully blocked by A438079. It shows that the P2X receptor expression is the same as we observed in the cell culture and that the cell culture is suitable to investigate receptor/channel expression in spermatogonia and Sertoli cells.

Additionally, this approach represents a huge advantage in studying the interplay between Sertoli cells and spermatogonia in their physiological environment.

### 4.2 Excursus 1: Deletion of GAR22$\beta$ Gene Impairs Spermatogenesis and Spermatozoa Motility

In collaboration with the institute of Biomedical Engineering at the University Clinic Aachen we investigated the function of the cytoskeletal linker protein GAR22$\beta$ in male reproduction.

The GAR22$\beta^{-/-}$ mice showed reduced male fertility. Our results indicate that this deficiency was caused by a significantly smaller fraction of motile sperm and broadly
impaired sperm ultrastructure (Gamper et al., 2015). Expression of a $\beta$-galactosidase reporter revealed a distinct staining pattern in the adluminal part of the tubules, indicating that GAR22$\beta$ is prominently expressed in post-meiotic germ cells. This result suggests that GAR22$\beta$ plays an important role in the development of spermatids, spermatocytes and possibly in spermatozoa. This finding is further supported by the fact that we did not detect any GAR22$\beta$ staining in testes from juvenile animals (P7), which do not yet show meiotic activity.

Our collaboration partners found that GAR22$\beta$ plays a role in dynamic structural interactions of the actin-MT cytoskeleton in Sertoli cells. These nurturing cells are essential for germ cell development. Accordingly, MT disruption caused dramatic effects such as increased apoptosis, detachment and abnormal germ cell positioning (O’Donnell and O’Bryan, 2014). In our laboratory, we analyzed the role of GAR22$\beta$ in postmeiotic germ cells, specifically focusing on ES and BTB structure. Both are important cytoskeletal components crucial for spermatogenesis (Vaid et al., 2007). We used immunolabeling in cryo-sections of seminiferous tubules. Our staining revealed a strong overlap of f-actin and GAR22$\beta$ in WT testes. The staining pattern was associated with the Sertoli cell structure. Moreover, the fluorescence intensity of f-actin was reduced in the GAR22$\beta^{-/-}$ tubules slices.

Taking into account that GAR22$\beta$ regulates MT function during spermatogenesis via its dual interaction with EB1 connected MTs and actin, the GAR22$\beta^{-/-}$ phenotype could be partially caused by changes in Sertoli cell function. Together, these findings indicate that reproductive capacity in male GAR22$\beta^{-/-}$ mice is impaired in multiple ways. Mechanistically, however, the relative contribution of either effect is not yet yet clear.

### 4.3 Excursus 2: CLARITY for Rapid Clearing and Imaging of Intact Anatomical Structures

CLARITY is a method to render whole-mount organs transparent in an active process to enhance imaging capabilities. We established this method published by Chung and Deisseroth (2013) in our lab because it has important advantages, compared to other clearing methods. CLARITY is the first method that allows complete clearing of entire organs within several days. We used clarity to prepare a variety of tissue samples. In figure 3.30 we present a 3D-reconstruction of a whole OMP-GFP P1 mouse head in order to visualize the peripheral olfactory system. We were able to obtain subcellular resolution even 2 mm deep in the tissue. This penetration depth exceeds all other published
methods, taking into account that the preparation included the whole head including cartilage and bone. With this preparation we were able to image the complex peripheral olfactory system as a whole. As shown in figure 3.30, axon bundles derive from the MOE and converge into the MOB. Previously, the analyses of this connectivity were tedious and included error-prone slice reconstruction.

One key feature of CLARITY is the preservation of intrinsic fluorophores. This allowed the analysis of numerous reporter mouse lines. Lee et al. (2014) showed clearing of various other tissues including pancreas, liver, kidney, lung and intestine. Also seminiferous tubules have been examined (Epp et al., 2015). Additionally, immune epitopes remain intact and the method increases the permeability for hydrophilic substrates. Passive diffusion of antibodies in 1 mm thick slices have been described as feasible (Chung et al., 2013). However, very long incubation times might be needed in samples exceeding 4 mm in diameter. If necessary we could expand our expertise and establish the electrophoretically driven immunolabeling of clarified tissues as described by Li and coworkers (Li et al., 2015).

We think this new method represents a major breakthrough in whole-mount organ analysis, as it exceeds many other approaches. A considerable downside, however, is its complexity and financial effort compared to other approaches. However, as we managed to establish the method, a variety of upcoming projects can be addressed easily.
Spermatogenesis is a fundamental biological process that ensures male fertility. However, few physiological details are known about testicular cell communication during spermatogenesis. Using wildtype C57BL/6 mouse pups, I first developed a co-culture of Sertoli cells and spermatogonia. Next, I investigated ATP-dependent signaling in spermatogonia by combining electrophysiological recordings with pharmacological profiling and protein expression knockdown. This approach allowed identification of those ATP-sensitive ion channels functionally expressed undifferentiated germ cells. I found that cultured spermatogonia respond to extracellular ATP (1–100 μM). ATP-induced currents show fast activation and moderate desensitization. The current–voltage relationship reveals strong inward rectification. Current potentiation by ivermectin and inhibition by an acidic extracellular pH (6.3) as well as extracellular copper (100 μM) indicate a functional role of P2X4 receptors. Accordingly, knockdown of P2X4 receptors (P2X4Rs) expression by RNA interference significantly reduced currents activated by ATP concentrations ≤ 300 μM. Interestingly, an increased ATP concentration (>300 μM) activated an additional current with different kinetics. A similar current could be activated by 300 μM BzATP and was blocked by the P2X7 antagonist A-438079. Similarly, knockdown of P2X7R expression decreased this current. Combined with molecular evidence, my results show the functional expression of at least two P2X receptor subunits (P2X7R and P2X4R) in spermatogonia of prepubescent mice. Downstream P2X receptor activation, I identified a calcium-dependent potassium current functionally antagonizing the depolarizing effect of P2XR activation. To confirm these results in situ, I established an acute tissue slices preparation from prepubescent mouse seminiferous tubules. Electrophysiological recordings from both Sertoli and germ cells revealed ATP-induced currents that strongly resembled my in vitro results.
Taken together, I established a toolkit to investigate transient responses from identified testicular cell types in a physiological setting. My data thus represent an important step towards a deeper understanding of cellular purinergic communication during spermatogenesis.

In a collaborative project, I investigated the function of the GAR22β gene in spermatogenesis. Knockout of the GAR22β protein leads to infertility in male mice. Spermatozoa are reduced in number, have impaired motility and display ultrastructural disorganization of spermatozoa. Moreover, Sertoli cells are affected by alterations in their microtubules and actin-dependent cytoskeleton dynamics. I showed that GAR22β is highly expressed in postmeiotic germ cells. GAR22β was colocalized with actin in testes and GAR22β-deficient mice showed reduced actin levels. This study revealed new insights into the poorly characterized function of the GAR22β protein and its involvement in spermatogenesis. The data of this project have been published in *Molecular Biology of the Cell* (Gamper et al., 2015).

I also established CLARITY, a method to render intact anatomical structures transparent to increase light penetration depth. Analyzing whole organs without sectioning allows analysis of complex structures in subcellular resolution.

Together, the data I acquired in this thesis provide important insights in physiological mechanisms of multiple facets of spermatogenesis. The methods I established during my thesis will facilitate a multitude of projects aimed to answer urgent questions in the field of reproductive sciences.
References


de Rooij DG, Russell LD (1997) All you wanted to know about spermatogonia but were afraid to ask. *Journal of andrology* 21:776–98.


References


Spalteholz W (1914) Über das Durchsichtigmachen von menschlichen und
tierischen Präparaten und seine theoretischen Bedingungen, nebst Anhang:
Über Knochenfärbung.

Steinberger E (1971) Hormonal Control of Mammalian Spermatogenesis. 
Physiol Rev 51:1–22.


Homodimeric anoctamin-1, but not homodimeric anoctamin-6, is acti-
vated by calcium increases mediated by the P2Y1 and P2X7 receptors. 
Pflugers Arch - Eur J Physiol.

Stoop R, Surprenant A, North RA (1997) Different sensitivities to pH of
ATP-induced currents at four cloned P2X receptors. Journal of neuro-
physiology 78:1837–40.

in the regulation of cell proliferation and differentiation. Stem cell re-
views 5:231–46.

Cytolytic P2Z Receptor for Extracellular ATP Identified as a P2X Receptor

Tan KAL, Turner KJ, Saunders PTK, Verhoeven G, De Gendt K, Atanassova
N, Sharpe RM (2005) Androgen regulation of stage-dependent cyclin D2
expression in Sertoli cells suggests a role in modulating androgen action on

Tanaka J, Murate M, Wang CzZ, Seino S, Iwanaga T, Sein S, Iwanaga T,
of the P2X4 ATP receptor mRNA in the brain and non-neuronal organs of


Abbreviations

$CO_2$ carbon dioxide

$\beta$ME beta-Mercaptoethanol

$\tau_{\text{pipette}}$ Time constant of the recording Pipette

$A_{\text{int}}$ Type A intermediate spermatogonia

ADP Adenosine 5’-triphosphate disodium

AM acetoxymethylester

AMP Adenosine 5’-triphosphate disodium

anti-BKCa Extracellular anti-KCa1.1

ATP Adenosine 5’-triphosphate disodium

BKCa $Ca^{2+}$-dependent potassium

BSA albumin from bovine serum

BTB blood-testis barrier

BzATP $2'(3')$-O-(4-Benzoylbenzoyl)adenosine 5’-triphosphate triethylammonium salt

$C_{\text{fast}}$ Recording pipette capacity

$C_{\text{slow}}$ Cell membrane capacity

CIB1 calcium and integrin binding 1

CLARITY Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue-hYdrogel

CP cribriform plate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>CS</td>
<td>Clearing Solution</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper(I) chloride</td>
</tr>
<tr>
<td>DAZL</td>
<td>deleted in azoospermia-like</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast</td>
</tr>
<tr>
<td>DIV</td>
<td>days <em>in vitro</em></td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Essential Medium</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco’s phosphate-buffered saline</td>
</tr>
<tr>
<td>E-NTPDase</td>
<td>Ectonucleoside triphosphate diphosphohydrolase</td>
</tr>
<tr>
<td>EC</td>
<td>half maximal effective concentration</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ES</td>
<td>ectoplasmic specialization</td>
</tr>
<tr>
<td>ETC</td>
<td>Electrophoretic Tissue Clearing</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>G protein</td>
<td>guanine nucleotide-binding</td>
</tr>
</tbody>
</table>
GA glutaraldehyde

GAR22β Gas2-related protein on chromosome 22

GnRH Gonadotropin-releasing hormone

GTP Guanosine 5’-triphosphate

HKG Housekeeping gene

HM Hydrogel monomer solution

HMDS Hexamethyldisilazane

HPG hypothalamic–pituitary–gonadal axis

HyD hybrid detector

IBTX Iberiotoxin

IP$_3$ inositol 1,4,5-triphosphate

IR-DIC infra-red differential interference contrast

ISI interstimulus interval

IV Current Voltage

LH luteinizing hormone

MEM Minimum essential media

MOB main olfactory bulb

MOE main olfactory epithelium

MT microtubules

NMDG N-methyl-d-glucamine

nRIMS60 Refractive index matching solution with 60% Nycodenz

P postnatal day

PBST PBS with TritonX
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PMT</td>
<td>Photomultiplier</td>
</tr>
<tr>
<td>PP</td>
<td>polypropylene</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphate buffer 200 mM</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative-PCR</td>
</tr>
<tr>
<td>RI</td>
<td>refractive index</td>
</tr>
<tr>
<td>RIMS</td>
<td>Refractive index matching solution</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA-Interference</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA-Interference</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning Electron Microscopy</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNAs</td>
</tr>
<tr>
<td>SSC</td>
<td>spermatogonial stem cells</td>
</tr>
<tr>
<td>TBST</td>
<td>Tris-Buffered saline with Tween 20</td>
</tr>
<tr>
<td>TEA</td>
<td>Tetraethylammonium</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylendiamin</td>
</tr>
<tr>
<td>TFM</td>
<td>Tissue Freezing Medium</td>
</tr>
</tbody>
</table>
Acknowledgment

