Unexpected salivary secretory effects of some "atypical" antipsychotics

- preclinical studies on clozapine, *N*-desmethylclozapine, amisulpride and olanzapine

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To Filomena

Abstract

Antipsychotics are generally associated with dry mouth and deterioration of the oral health. However, clozapine, the archetype of the atypical antipsychotics, is reported to induce not only mouth dryness but also, in about one-third of the patients, hypersalivation, the latter resulting in disturbed sleep, coughing and choking sensations during the night and drooling during the day. Nevertheless, the hypersalivation is questioned and, in some studies, related to a weakened swallowing reflex. Clinical studies are inconclusive and based on subjective drooling scores and indirect measurements of the saliva secreted. Preclinical studies on the effect of clozapine on the salivary flow are lacking. The aim of this *Thesis* was to explore the salivary secretory role of some atypical antipsychotics in an animal model, with clozapine-induced sialorrhea in focus. A secretory role for clozapine and its metabolite *N*-desmethylclozapine was established: saliva was secreted from duct-cannulated submandibular and parotid glands in the rat. The action was direct, independent on circulatory catecholamines and nerves, and mediated via muscarinic M_1 receptors. Together, the weaker agonist clozapine prevented its metabolite from exerting full agonistic effect. Thus, the sialorrhea in the clinic may be explained by a continuous bombardment of muscarinic M_1 receptors. At higher demands on the flow-rate, such as during a meal, the patient is, however, likely to experience insufficient salivation due to the clozapine/*N*-desmethylclozapine blockade of muscarinic M_3 and α_1 adrenergic receptors. Since clozapine/*N*-desmethylclozapine did not antagonize the β_1 adrenergic receptor, a sympathetic β_1-mediated salivary response can be expected to add to the muscarinic M_1 -mediated response during daytime; moreover stimulation of the two receptor types interacted positively. The antipsychotic drug amisulpride, reported to abolish the clozapine-induced sialorrhea, failed in the preclinical model. In contrast, it potentiated the secretory response to nervous activity as well as to autonomimetics, without causing secretion *per se*. Amisulpride exerted its effect at gland level but the mechanism is currently unknown.

Amisulpride may be a potential drug for dry mouth treatment. Olanzapine, with a reported receptor profile similar to that of clozapine, evoked secretion, like clozapine but by other receptors, involving the substance P-type. In human salivary glands, acini but not vessels, lack substance P innervation. Therefore, olanzapine, in the clinic, is not a secretagogue via this receptor but may cause vasodilation and oedema formation as a part of an inflammatory response.

Keywords: schizophrenia, atypical antipsychotics, sialorrhea, clozapine-induced sialorrhea, clozapine, *N*-desmethylclozapine, amisulpride, olanzapine, salivary secretion, muscarinic acetylcholine receptors, adrenergic receptors, non-adrenergic, non-cholinergic receptors, tachykinins

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Populärvetenskaplig Sammanfattning

Behandling med antipsykotiska läkemedel förknippas vanligtvis med uttalad muntorrhet och destruerad munhälsa. Överraskande nog föreligger kliniska rapporter som framhäver ökad salivation, hos en tredjedel av patienterna, som svar på clozapinbehandling medan andra säger sig vara muntorra. Under natten besväras patienten av störd sömn, hosta och kvävningsattacker och under dagtid av dregling. Salivsekretionen kan bli så besvärande att behandlingen får avbrytas. Clozapin, ett så kallat atypiskt antipsykotikum, används vid behandling av schizofreni. Läkemedlet har en överlägsen terapeutisk profil då det dämpar symptom som hallucinationer, vanföreställningar, initiativlöshet, känslomässig förflackning och inåtvändhet samtidigt som påverkan av motoriken (extrapyramidala biverkningar, såsom parkinsonism) undvikes, det senare utmärkande för första generationens antipsykotika. Trots rapporterna om ökad salivation under clozapinbehandling ifrågasätts fenomenet. Vissa hänför salivationen till en försvagad sväljningsreflex snarare än till en faktisk ökning av salivproduktionen. Prekliniska studier över clozapinets verkan på salivflödet saknas och konklusionerna från de kliniska studierna är motstridiga. Att förekommande kliniska studier är baserade på patientens subjektiva värdering och inte på objektiva mätningar av salivflödet försvårar tolkningen av den clozapinutlösta salivationen ytterligare. Flera läkemedelskategorier har prövats med målet att hämma den clozapinutlösta salivationen, bland annat det atypiska antipsykotiska medlet amisulprid. Även så drastiska metoder som att skära av salivkörtelnerverna har föreslagits.

Aktuell avhandling syftar till att erbjuda en djurexperimentell förklaringsmodell till klinikens clozapininducerade salivation samt att erbjuda en vetenskaplig grund till behandling av densamma. I detta sammanhang uppmärksammas effekten av clozapinets metabolit, *N*-desmetylclozapin, och dess samverkan med modersubstansen.

Mot bakgrund av att amisulprid föreslagits till att bemästra clozapinutlöst sekretion så prövas denna substans i djurmodellen. Med anledning av att clozapin kan ge upphov till en allvarlig biverkan på blodbilden har ett antal clozapinlika ämnen, vilka saknar denna biverkan, introducerats varav ett är olanzapin.

Clozapin och *N*-desmetylclozapin visade sig ha blandade effekter på salivsekretionen. Substanserna stimulerade körtelcellerna via kolinerga muskarin M_1 receptorer samtidigt som de förhindrade nervutlöst sekretion via blockad av cellernas muskarin M_3 och α_1-adrenerga receptorer. *N*-desmetylclozapins stimulerande effekt visade sig vara större, och dess hämmande effekt mindre, än clozapinets. Samtidigt närvarande, hindrade det mindre potenta sekretionsstimulerande clozapin, metaboliten från att utöva sin fulla effekt. Experimenten gav inga belägg för att amisulprid verkade hämmande på den clozapinutlösta salivationen. Inte heller gav amisulprid upphov till något salivflöde i sig. Istället, och oväntat, potentierade substansen en redan pågående salivation utlöst via nervaktivitet eller sekretionsframkallande substanser. Olanzapin visade sig, likt clozapin, utlösa ett salivflöde. Till skillnad från clozapinet förmedlades sekretionen via andra receptorer, så kallade icke-adrenerga, icke-kolinerga receptorer, delvis av substans P typ. Avhandlingens resultat bekräftar förekomsten av en clozapinutlöst salivsekretion och lämnar en förklaring till både en sekretions-framkallande och sekretionshämmande effekt. Under natten, då endast de små salivkörtlarna svarar för salivsekretionen, adderas den clozapin-/*N*-desmetylclozapinutlösta salivsekretion från små och stora körtlar, vilket ger upphov till den störda sömnen. När det under dagtid ställs större krav på salivsekretionen kommer blockaden av muskarin M_3 receptorn och den α_1-adrenerga receptorn att ta överhand, vilket medför sänkt reflex-utlöst sekretion. Amisulprids förstärkande effekt kan man tänkas använda som utgångspunkt för utvecklingen av läkemedel mot muntorrhet. Olanzapin har sannolikt ingen salivstimulerande effekt via substans P receptorer hos människa, eftersom människans sekretoriska salivkörtel-celler saknar denna typ av receptor. Emellertid är körtlarnas kärl försedda med substans P receptorer, vilka kan tänkas stimuleras av olanzapin och därmed understödja en inflammation med kärlvidgning och ödembildning, något som även kan tänkas ske i andra organ i kroppen.

Original Publications

This *Thesis* is based on the following studies, referred to in the text by their Roman numerals.

I. **Clozapine: agonistic and antagonistic salivary secretory actions**
Ekström, J. Godoy, T. Riva, A
Journal of Dental Research 2010; 89: 276-280.

II. ***N*-desmethylclozapine exerts dual and opposite effects on salivary secretion in the rat**
Ekström, J. Godoy, T. Riva, A
European Journal of Oral Sciences 2010; 118: 1-8.

III. **Clozapine-induced salivation: interaction with *N*-desmethylclozapine and amisulpride in an experimental rat model**
Godoy, T. Riva, A. Ekström, J
European Journal of Oral Sciences 2011; 119: 275-281.

IV. **Atypical antipsychotics - effects of amisulpride on salivary secretion and on clozapine-induced sialorrhea**
Godoy, T. Riva, A. Ekström, J
Oral Diseases 2012; 18: 680-691

V. **Salivary secretion effects of the antipsychotic drug olanzapine in an animal model**
Godoy, T. Riva, A. Ekström, J
Oral Diseases 2013; 19: 151–161

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Introduction

The phenomenon of clozapine-induced sialorrhea

Antipsychotic therapy of schizophrenia is usually associated with mouth dryness and deterioration of the oral health. However, clozapine, the archetype of atypical antipsychotics differs from the general pattern. It is reported to evoke mixed salivary secretory actions: patients may complain of either dry mouth (Scully and Bagan, 2004, McEvoy et al., 2006) or sialorrhea (Chengappa et al., 2000, McEvoy et al., 2006, Praharaj et al., 2010). Indeed, about one-third (23-50%) of the individuals treated with clozapine are troubled by embarrassing drooling during the daytime and disturbed sleep during the night due to coughing, choking sensations and the aspiration of saliva (McEvoy et al., 2006, Praharaj et al., 2006). The side effects can be so bothersome that, despite good treatment results of the disease, the clozapine regimen has to be discontinued. Even though there are several reports of a clozapine-induced sialorrhea, the phenomenon is questioned. The idea of a hypersalivation in the clinical situation is based on various subjective drooling scores, and indirect measurements, such as the wetted area of the pillow or the weight of collected saliva in bibs, rather than on the actual measurement of the salivary secretion (Praharaj et al., 2006). Moreover, no preclinical studies on the effect of clozapine on the actual flow of saliva are on record. In fact, some investigators ascribe the "sialorrhea" to a neuromuscular inhibitory effect of clozapine, weakening the swallowing reflex, allowing saliva to be pooled in the anterior part of the mouth and then to run over the lips (McCarthy and Terkelsen, 1994, Pearlman, 1994, Rabinowitz et al., 1996). Since the cause of the clozapine-induced sialorrhea is unknown and its existence even debated, a rational ground for its treatment is lacking. Due to its superiority in the treatment of schizophrenia, an interruption of the clozapine therapy is unwanted. Therefore, a number of drugs have been clinically tried in an attempt to abolish the "sialorrhea", with varying success and with side effects of their own. Among the tested drug categories are muscarinic receptor antagonists, α_1-

adrenergic-receptor antagonists, β-adrenergic-receptor antagonists, α_2-adrenergic-receptor agonists, histamine$_1$-receptor antagonists, tricyclic antidepressants, botulinum toxin and recently benzamide derivates such as amisulpride. Among surgical approaches considered to abolish the clozapine-induced sialorrhea is the cutting of the salivary nerves (Rabinowitz et al., 1996, Sockalingam et al., 2007).

At the start of the current *Thesis* work, the phenomenon of clozapine-induced sialorrhea, reported in clinical investigations, was largely enigmatic. Turning to the few *in vitro* studies, indirectly displaying secretory activity, the results gave inconsistent data. Whereas ultrastructural changes, evoked by clozapine in pieces of human submandibular gland tissue, gave support for the idea of a secretory role of clozapine (Testa Riva et al., 2006), no support for such a role of clozapine was gained from a study on isolated rat submandibular acinar cells, measuring intracellular Ca^{2+} (Pochet et al., 2003).

This *Thesis*, objectively recording the flow of saliva from duct-cannulated glands in an animal experimental model, demonstrated both excitatory and inhibitory effects on the secretion in response to the administration of clozapine (I). Moreover, the present *Thesis* showed *N*-desmethyl-clozapine, the main active metabolite of clozapine, to display a similar secretory pattern as the parent substance, although the efficacy varied between the drugs (II). The two drugs did not interact synergistically, since the combined volume response of saliva was less than the sum of the response induced by each drug (III). Amisulpride, clinically reported to attenuate the clozapine-induced saliva, was without effect on the flow of saliva evoked by clozapine in the animal model. Nevertheless, amisulpride, also belonging to the class of atypical antipsychotics, was found to act as a "secretory amplifier" of the response to nerve stimulation and administration of autonomic receptor agonists (IV). Finally, olanzapine, yet another atypical antipsychotic thought to act on the same set of receptors as clozapine, was tested and found to evoke secretion like clozapine, but by other receptors than clozapine (V).

Schizophrenia and antipsychotics

Schizophrenia affects about one per cent of the population (Meyer, 2011). The onset of symptoms often occur in young adulthood (Lewis and Lieberman, 2000) and the vulnerability for the disease is partly genetic and partly due to environmental factors (van Os and Kapur, 2009). Schizophrenic psychosis is characterized by positive and negative symptoms. Positive symptoms include hallucinations, delusions and thought disorders, whereas negative symptoms include cognitive deficits, affective flattening, monosyllabic speech and withdrawal from social contacts. To diagnose schizophrenia the US- based 4th Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) and the 10th International classification of Diseases (ISD-10) are used.

Chlorpromazine, the first antipsychotic drug, introduced in the 1950s revolutionized the treatment of schizophrenia. The patients became calm and sedated concomitant with alleviation of their psychotic symptoms (Swazey, 1974, Shen, 1999). Chlorpromazine was followed by haloperidol (1958), which is still in use. The first generation of antipsychotics is associated with marked extrapyramidal side effects such as parkinsonism (rigidity, tremor and bradykinesi), acute dystonia and akathisia and, at a late stage, tarditive dyskinesia. Moreover, the treated patient is troubled with galactorrhea, ortostatic dizziness and dry mouth and further; the negative symptoms are often resistant to the therapy. In the search of antipsychotic drugs with less extrapyramidal side effects, clozapine, a benzodiazepine, reached the market in the early 1970s. With effectiveness on both positive and negative symptoms combined with lack of extrapyramidal side effects, clozapine became the prototype for the second generation of antipsychotics, also called "atypical" antipsychotics, in opposite to the "typical" ones of the first generation (Gardner and Teehan, 2011) However, in the middle of the 1970s, clozapine was withdrawn from the market in some countries due to its serious side effect of agranulocytosis. About fifteen years later (1989), the drug re-appeared on the international market due to its therapeutic advantage on therapy-resistant schizophrenia. However, this time with mandatory monitoring of the blood. Due to its hematological side effect, clozapine is not the first hand choice in the therapy.

The effectiveness of clozapine in the treatment of schizophrenia initiated the development of other atypical antipsychotics with a safer profile than clozapine with regard to agranulocytosis, such as the substituted benzamide sulpride (1967) later followed by amisulpride, and the thienobenzodiazepine olanzapine (1996).

Antipsychotic drugs display various receptor profiles and although the reason for the beneficial effect of antipsychotics in the treatment of schizophrenia is debated (Gründer et al., 2009), the common denominator is the antagonistic effect exerted on dopamine D_2 receptors (Carlsson, 1978, Meyer, 2011). The improved therapeutical profile of the atypical antipsychotics has tentatively been attributed to their greater affinity for blocking serotonin receptors of 5-HT_2 type than for blocking dopamine D_2 receptors (Meltzer et al., 1989). Clozapine and olanzapine exert antagonistic effect on serotonin receptors and further, clozapine and amisulpride dissociate more rapidly from D_2 receptors than the typical antipsychotics (Seeman and Tallerico, 1999). Clozapine is continuously metabolized in the liver and intestines to *N*-desmethylclozapine. *N*-desmethylclozapine was tentatively suggested for the positive therapeutic effect of clozapine, attributed to a partial D_2 receptor agonism as well as to a muscarinic M_1 receptor agonism (Lameh et al., 2007). However, when subjected to clinical trials, *N*-desmethylclozapine failed as monotherapy for schizophrenia (Meyer, 2011)

Although, a high ratio 5-HT_2/D_2 is in focus with respect to the effectiveness of the atypical antipsychotics in the treatment of schizophrenia, it should be realized that antipsychotic drugs also display affinity for additional receptor subclasses or classes. Clozapine is considered the most anticholinergic drug of all atypical antipsychotics (Gardner and Teehan, 2011). Yet a partial agonistic effect on muscarinic M_1/M_4 receptors has, in some studies, been ascribed to clozapine (Ashby and Wang, 1996, Davies et al., 2005). Moreover, it is an antagonist to α_1- and α_2- adrenergic receptors and to histamin H_1 receptors (Ashby and Wang, 1996, Gardner and Teehan, 2011). *N*-desmethylclozapine is not only a weak agonist to dopamine D_2 but also to D_3 receptors. It is a potent (partial) agonist to the muscarinic M_1 receptor.

N-desmethylclozapine shows less affinity to α-adrenergic receptors than clozapine, but nevertheless the affinity is characterized as high. The affinity for histamine H_1 receptors is similar to that of clozapine, but functionally *N*-desmethylclozapine is reported as less potent than clozapine (Lameh et al., 2007, Davies et al., 2005, Snigdha et al., 2010). Olanzapine shows, on the whole, a similar receptor profile as clozapine but seems to lack any agonistic effect on muscarinic M_1 receptors (Davies et al., 2005, Theisen et al., 2007, Meyer, 2011). Amisulpride, in contrast to the three other drugs, is a specific dopamine antagonist that displays a high and selective affinity for the dopamine D_2 and D_3 receptor (Schoemaker et al., 1997, Rosenzweig et al., 2002, Pani and Gessa, 2002). Moreover, at a low dose, amisulpride preferentially blocks presynaptic dopamine D_2/D_3 receptors, whereas at higher doses, it blocks postsynaptic D_2/D_3 receptors. The dual actions have been attributed to the improvement of the negative symptoms of schizophrenia at a low dose, and the improvement of the positive symptoms at a high dose. An antagonistic effect of amisulpride on 5-HT_7 receptors has been demonstrated and suggested to be involved in the improvement of depression in response to amisulpride treatment (Abbas et al., 2009).

According to The National Board of Health and Welfare, about 5 800 patients were treated with clozapine in Sweden in 2012, as compared to about 30 200 treated with olanzapine and 14 800 patients treated with the typical antipsychotic drug haloperidol (Socialstyrelsen, 2013). Amisulpride is not marketed in Sweden. Only twenty-two patients were treated with the related drug sulpiride in 2012.

Receptors in salivary glands mediating secretion

Muscarinic receptors

Muscarinic receptors are divided into five subtypes, M_1-M_5 (Hammer et al., 1980, Bonner, 1989, Caulfield, 1993, Caulfield and Birdsall, 1998, Eglen, 2012). The muscarinic M_1, M_2 and M_3 receptors are excitatory as they increase intracellular calcium, while the M_2 and M_4 receptors are inhibitory as they inhibit the adenylate cyclase activity (Ben-Chaim et al., 2003). Acetylcholine binds to the orthosteric site of the receptor; in

addition, other compounds may bind to the various allosteric sites on the receptor, to modulate the agonistic function (Mohr et al., 2003, Voigtlander et al., 2003, Wess, 2005, Conn et al., 2009, Eglen, 2012).

Activation of muscarinic receptors plays a pivotal role in the secretion of saliva. In salivary glands in man as well as in animal species, all five receptor types have been demonstrated to a varying extent and localization by techniques such as radio-ligand binding, immunohistochemistry and immunoblotting (Abrams et al., 2006, Khosravani et al., 2007, Ryberg et al., 2007, Tobin et al., 2009). A functional salivary secretory role, as judged from animal experiments, is generally ascribed to muscarinic M_1 and M_3-receptors, expressed postjunctionally in the basal membrane of the acinar cell, with predominance for the M_3 receptor-mediated response (Tobin, 2002, Bymaster et al., 1996, Takeuchi et al., 2002, Nakamura et al., 2004, Gautam et al., 2004, Ryberg et al., 2007, Tobin et al., 2009); a (minor) role for M_4 and M_5 receptors has been implied as well but not convincingly shown. Prejunctionally, muscarinic receptors of subtypes M_2 and M_4 are considered to inhibit the transmitter release, while receptors of subtype M_1 is considered to facilitate the transmitter release from the cholinergic nerve endings (Abrams et al., 2006, Eglen, 2012), mechanisms that seems to be at work in salivary glands (Tobin et al., 2009).

Adrenergic receptors

The α-adrenergic and β-adrenergic receptors of functional importance for the salivary secretion belong to the α_1- and β_1- subtypes (Emmelin, 1965, Ekström, 1969, Thulin, 1972, Au et al., 1977, Pointon and Banerjee, 1979, Bylund and Martinez, 1980, Jensen et al., 1991). The relative contribution to the volume response by α-adrenergic receptors and by β-adrenergic receptors, respectively, varies between glands and species (Emmelin, 1981). For instance, in the cat parotid gland the β-mediated volume response dominates (Ekström and Emmelin, 1974), while in the rat parotid gland the α-mediated volume response dominates (Ekström, 1980). The two types of receptors are responsible for different qualities of the saliva produced, depending on the various intracellular pathways mobilized. The α-mediated response activates the inositol triphosphate/

Ca^{2+}-pathway, like the muscarinic receptors, leading to watery and enzyme rich saliva. The β-mediated response activates the cyclic adenosine monophosphate (cyclic AMP) leading to a protein rich, less watery saliva.

Non-adrenergic, non-cholinergic receptors

Besides the receptors for the classical transmitters acetylcholine and noradrenaline, the salivary glands are also supplied by so called non-adrenergic, non-cholinergic receptors (Ekström, 1999b). Retrospectively, at a time long before chemical transmission was recognized, the first example of non-adrenergic, non-cholinergic transmission on record is the observation of Heidenhain in 1872 of an atropine-resistant vasodilatation in the submandibular gland of the dog upon stimulation of the parasympathetic innervation. In contrast, the early pioneers of physiology found in their studies on cats and dogs, the copious flow of saliva in response to stimulation of the parasympathetic innervation to be easily abolished by atropine, giving rise to the general idea that the parasympathetic-evoked secretion of saliva is completely atropine-sensitive and thus, only depending on cholinergic transmission. About a century later, vasoactive intestinal peptide released from the parasympathetic nerves was shown to play a major role in mediating the vasodilator response (Bloom and Edwards, 1980, Lundberg et al., 1980). Moreover, at about that time and after turning to other species than cats and dogs, such as the rat and the ferret, parasympathetic atropine-resistant secretion, albeit reduced, was demonstrated (Thulin, 1976, Ekström et al., 1983). A variety of neuropeptides of parasympathetic origin (vasoactive intestinal peptide, pituitary adenylate activating petide, calcitonin gene-related peptide, neuropeptide Y and the tachykinins substance P and neurokinin A) is involved in a number of glandular activities apart from causing the secretion of fluid and proteins, such as gland metabolism and gland growth (Ekström, 1999b). Importantly, the non-adrenergic, non-cholinergic transmitters are at work upon parasympathetic stimulation also in those glands, which lack an overt fluid secretion in response to the nerve stimulation. For instance upon parasympathetic activity, the cat parotid gland loses its acinar content of

secretory granules and protein is secreted. Vasoactive intestinal peptide induces a small flow of saliva rich in protein, as in the rat parotid gland, or just protein release with no overt volume response, as in the cat parotid gland. On the other hand, substance P induces a rich flow of saliva but only in some species, e.g., in the rat but not in the cat. In human glands vasoactive intestinal peptide-containing nerve fibres occur close to the acinar cells, while the acinar cells lack a substance P-innervation (Hauser-Kronberger et al., 1992). In agreement, substance P is without effect on the potassium release from pieces of the human submandibular gland, whereas vasoactive intestinal peptide increases the cyclic AMP level in the gland tissue (Larsson et al., 1986). Though nitric oxide is found in the parasympathetic nerves of the glands and thought of as a transmitter (Alm et al., 1995), nitric oxide of non-neuronal origin seems rather to be associated with a number of glandular events linked to sympathetic activity (and cyclic AMP) induced protein secretion, protein synthesis and mitotic activity (Sayardoust and Ekström, 2003, Ekström et al., 2004, Sayardoust and Ekström, 2004, Sayardoust and Ekström, 2006, Aras and Ekström, 2008).

In addition, some further regulatory mechanisms have been discussed but their relevance in salivary gland physiology is not clear. The purinergic receptor $P2X_7$ has upon activation by ATP been found to elicit flow of saliva from the perfused mouse submandibular gland. Moreover, the administration of ATP inhibited the muscarinic-induced fluid secretion from the gland. Whether the physiological source of ATP would be neuronal or parenchymal is unknown (Nakamoto et al., 2008).

In insects such as the blowfly and the cockroach, serotonin and dopamine cause secretion of saliva (Berridge, 1970, Baumann et al., 2004), while in mammals the roles of these substances in salivary glands are less certain. Serotonin *per se* causes no secretion from the rat parotid gland. It was in rats, however, reported to enhance the acetylcholine-evoked flow from this gland and to reduce the acetylcholine-evoked flow from the submandibular gland (Chernick et al., 1989, Turner et al., 1996). With respect to dopamine, the action is far from clear. In the rat parotid gland, dopamine may act indirectly, prejunctionally, by the release of noradrenaline and acetylcholine and/or directly on postjunctional

dopamine D_1 receptors (Sundström et al., 1985, Hata et al., 1986, Michalek and Templeton, 1986, Danielsson et al., 1988).

Cholecystokinin has been suggested as a parasympathetic non-adrenergic, non-cholinergic transmitter responsible for the atropine-resistant fluid secretion in the rat submandibular gland (Takai et al., 1998). Salivary glands are supplied with CCK-A and CCK-B receptors for cholecystokinin and gastrin (Cevik Aras and Ekström, 2006). Though, cholecystokinin-containing nerve fibres have been demonstrated in intestines and the pancreatic gland (Larsson and Rehfeld, 1979a, Larsson and Rehfeld, 1979b) no evidence for an action of cholecystokinin of nervous origin was found in the rat parotid gland upon parasympathetic stimulation (Cevik Aras and Ekström, 2006); the finding of Takai and co-workers (1968) may be explained by the depletion of the neuropeptide content upon prolonged stimulation rather than by the effect of a cholecystokinin-receptor blocker. Salivary glands may also be supplied with receptors for melatonin of both subtype 1 and 2, as shown in rats (Ekström and Cevik Aras, 2008). Cholecystokinin and gastrin are probably released from the gastro-intestinal tract, as may also be the case for melatonin, in response to a meal. The three peptide hormones cause salivary protein secretion and protein synthesis.

Finally it should be mentioned that histamine may evoke a scanty and irregular secretion of saliva, at high doses, most likely due to an indirect action of the substance (Emmelin, 1966). In the dog submandibular gland, the secretion of saliva evoked via histamine H_1 receptors is entirely dependent on excitation of parasympathetic postganglionic nerves, and is completely abolished by an atropine-like drug (Shimizu and Taira, 1980).

The functions of saliva

Saliva serves several purposes. It protects the oral structures by lubrication with mucins, cleanses the oral cavity, dilutes hot, cold or spicy food, maintains neutral pH by buffering with bicarbonate, phosphates and proteins, remineralizes the tooth surface (enamel and dentine) of the teeth with calcium, exerts an antimicrobial defence by immunoglobulin A, α-defensins, and β-defensins, and is involved in wound healing by growth

hormone, statherines, and histatines. Further, saliva has important digestive functions including facilitating mastication, bolus formation and swallowing, as well as chemical degradation of food by means of amylase and lipase, and dissolution of tastants (Kaplan and Baum, 1993, Ekström et al., 2012).

Saliva is derived mainly from the three pairs of major salivary glands, the parotid, the submandibular and the sublingual glands, located outside the mouth and with their excretory ducts entering the oral cavity. Additionally, hundreds of minor salivary glands are distributed throughout the oral mucosa just below the oral epithelium and they empty their saliva directly into the mouth via short excretory ducts. Of the major salivary glands in humans, the serous parotid gland and the seromucous submandibular gland are large glands, while the size of the mucous sublingual gland is much less. Most of the minor glands are mucous ones. The serous acinar gland cells produce saliva that mainly contains water and enzymes, while the mucous acinar cells produce a Mucin-rich film that covers the oral structures, preventing the sensation of dry mouth. Over 24 hours, 1-2 liter of saliva is secreted. The volume secreted depends on age, gland size and gender, men secreting more saliva than women (Heintze et al., 1983). The parotid gland contributes with about 30% of the volume of saliva secreted, the submandibular gland with 60%, the sublingual gland and the minor glands with 5% of each (Dawes and Wood, 1973). Notably, the minor glands secrete day and night, while the major glands are usually associated with the intake of food. In the mouth, the secreted saliva is mixed with bacterial products, food debris and exfoliating oral mucosal cells to form what is called whole saliva; of the about 2400 different proteins of whole human saliva characterized by proteomics, only one-tenth are of glandular origin (Ekström et al., 2012).

The secretory unit consists of acini and ducts. In the acini primary isotonic saliva is produced which is modified through its passage in the intraglandular duct system; sodium and chloride are reabsorbed without accompanying water, while potassium and bicarbonate are secreted but at a lower rate, resulting in secondary hypotonic saliva entering the oral cavity. Moreover, proteins and peptides are secreted both from acini and

ducts. Myoepithelial cells embrace acini and ducts. Upon contraction of the myoepithelial cells, the intraductal pressure increases, thereby facilitating the flow of viscous saliva (Garrett and Emmelin, 1979, Ekström et al., 2012).

To maintain salivary flow rates over a certain period of time the glands depend on the blood flow supplying the glands with water and solutes. The blood flow through a salivary gland may under a lively secretion increase twenty-fold. The gland has a dense network of blood vessels, being among the highest in the body and comparable to the heart (Edwards, 1998, Samje, 1998).

Nervous and hormonal regulatory mechanisms

Nervous activity is usually made responsible for the acute secretory response of fluid and proteins. Both nerves and hormones, such as the sex steroids, exert a long-term influence on gland size and structure, thereby indirectly influencing the secretory capacity. However, recent animal experiments suggest that the secretory response to a meal is not only evoked by nerves under a cephalic phase, but also by hormones under a gastric phase (gastrin) and an intestinal phase (cholecystokinin and melatonin) with respect to proteins (Cevik Aras and Ekström, 2006).

Parasympathetic nerves invariable innervate the secretory cells of the salivary glands, while the sympathetic innervation varies between different species and between glands in the same species (Emmelin, 1967); in humans the minor glands, as judged by the labial glands, lack a secretory sympathetic innervation (Rossoni et al., 1979). Notably, in salivary glands both the parasympathetic and sympathetic innervations act synergistically to cause secretion. Upon parasympathetic activity a high flow rate of saliva is produced. Providing the presence of sympathetic secreto-motor fibres, sympathetic activity gives rise to a low flow rate of saliva. With respect to the protein output, expressed in terms of concentration, sympathetic saliva is characterized as protein-rich and parasympathetic saliva as protein-poor. The sympathetic activity is thought to occur in a background of parasympathetic activity (Emmelin, 1987).

In humans the minor glands secrete spontaneously during the night, in the absence of nervous influences, while during the daytime a nervous drive adds to their continuous secretion. The type of gland responsible for the spontaneous secretion varies among species e.g., sublingual glands in the rat and the cat, and submandibular glands in the rabbit (Emmelin, 1967). At rest, a slow flow of saliva, mainly from the submandibular glands, is maintained by movements of the lips and the tongue. Eating is a strong stimulus for a range of receptors (mechanoreceptors, gustatory receptors, olfactory receptors and nociceptors) giving rise to a rich salivary secretion (Hector and Linden, 1999).

Dry mouth

Dry mouth refers to the oral sensation of dryness (xerostomia), with or without salivary gland hypofunction. The subjective feeling of dryness does not always correlate with an actual hypofunction of the salivary glands (Fox et al., 1987). In fact only about 55% of the patients complaining of dry mouth show a decrease in salivary secretion when objectively measuring the volume of saliva (Longman et al., 1995, Field et al., 1997). Unstimulated flow rate of whole saliva less than 0,1 ml per min, and a stimulated flow rate of whole saliva less than 0,7 ml per min are defined as hyposalivation (Ericsson and Hardwick, 1978). The expression sialorrhea refers to the contrary, that is hypersalivation. The term drooling is used when saliva runs over the lips. It can be caused by several conditions (e.g. neurological diseases, cerebral palsy and Parkinson's disease) thus it is not necessarily a consequence of an increase in salivary flow rate.

About 15-40% of the population is affected by dry mouth (Österberg et al., 1984, Nederfors et al., 1997). Among known causes; Sjögren´s syndrome, diabetes mellitus, depression, head and neck radiotherapy, radioiodine therapy, HIV/AIDS, orofacial trauma, surgery, and medications. Hyposalivation dramatically impairs the oral health. It is associated with caries, dysgeusia, dysphagia, gingivitis, halitosis, mastication problems, mucositis, candidiasis, speech difficulties and poorly fitting prostheses (Nederfors et al., 1997, Ship et al., 2002).

The experimental animal model

The rat and its salivary glands has served as model for experimental studies on secretion and various neurobiological phenomena over several decades at the laboratory, from where the current work originates. The various types of salivary glands are unique. Therefore, to explore different aspects of the autonomic nervous system and its regulation of salivary glandular activities one type of gland may serve a particular purpose better than another type of gland. To exemplify with respect to the parotid and the submandibular gland, and to the current experimental work: the volume responses to muscarinic and adrenergic agonists as well as to tachykinins are larger from the submandibular glands than from the parotid glands (per unit weight); and the volume response of the submandibular gland to stimulation of the sympathetic innervation is larger than from the parotid gland; the parasympathetic postganglionic innervation of the parotid gland is accessible for electrical stimulation while in the submandibular gland the relay between pre- and postganglionic parasympathetic nerves are located within the parenchyma allowing only for stimulation of the parasympathetic preganglionic innervation; the parotid gland can be parasympathetically post-ganglionically denervated, while the submandibular gland can only easily be parasympathetically preganglionically denervated. Both glands can, however, be subjected to sympathetic postganglionic denervation; and for studies on the glandular blood flow, the venous drainage of the submandibular gland is easier to collect than that of the parotid gland.

Eventually, *in vivo* experiments on one hand and *in vitro* experiments on the other hand have their particular advantages and disadvantages. In the *in vivo* situation the flow of saliva can be estimated, while *in vitro* one has to rely upon some indirect parameter to indicate fluid secretion. However, *in vitro* whole dose-responses can usually be constructed, while high doses *in vivo* may cause fatal systemic effects. Evidently, observations on reflex stimulation of the glands as well as of stimulation of the two branches of the autonomic nervous system, and the recording of the blood flow are limited to observations *in vivo*.

Aims

Since the clinical studies on the secretory actions of clozapine concern the volume of saliva rather than its composition, the volume response was in focus of the current *Thesis*.

In summary, the objective of this *Thesis* was to explore the salivary secretory role of some atypical antipsychotics in an experimental animal model, with particular reference to the clozapine-induced sialorrhea. Thus along the course of this doctoral work attentions were paid to define:

- the mixed agonistic/antagonistic secretory action of clozapine
- the secretory profile of the clozapine metabolite *N*-desmethylclozapine
- the interaction between *N*-desmethylclozapine and its parent compound
- the action of amisulpride on the salivation evoked by clozapine, nervous activity and secretagogues
- the action of olanzapine in comparison with that of clozapine

Materials and Methods

Animals

Adult female Sprague-Dawley rats (Charles River, Sulzfeld, Germany) maintained on a standard pelleted diet was used. The experiments were approved by the Ethics Committee for Animal Experiments in Gothenburg, Sweden. The guidelines established by the National Institute of Health (NIH) in the USA regarding the care and use of animals for experimental procedures were followed. The animals were anaesthetized with sodium pentobarbitone (25 mg/kg intraperitoneal) combined with ketamine (50 mg/kg intramuscular) for preliminary surgery or studies on the reflex stimulation. For the acute experiment, the animals were anesthetized with pentobarbitone (55 mg/kg, intraperitoneal, followed by supplementary intravenous doses as required). The body temperature of the anesthetized animal, measured using a rectal probe, was maintained at about 38°C using a thermostatically controlled blanket. The animals, still under anesthesia, were killed by exsanguination or an overdose of pentobarbitone. Glands were removed, cleaned, pressed gently between gauze pads and weighed.

Preliminary surgery

Preliminary surgery was performed 2-4 weeks in advance of the acute experiment to allow for nerve degeneration and the development of supersensitivity (Emmelin, 1952, Ekström, 1980, Ekström, 1999a). Combined parasympathetic and sympathetic postganglionic denervation of the parotid gland was done by the avulsion of the auriculo-temporal nerve, identified where it emerges from the base of the skull, and of the superior cervical ganglion (Alm and Ekström, 1977, Khosravani et al., 2006) (I-V). Preganglionic parasympathetic denervation of the submandibular gland was achieved by cutting the chorda-lingual nerve (I, II, III). Surgery was performed unilaterally.

Terminal surgery

The anaesthetized animals were fitted with a femoral venous polyethylene catheter, which served as a conduit for injections of drugs and/or saline, and a tracheal cannula (I-V). The parotid duct was exposed externally by an incision in the cheek, close to its entrance into the mouth, while the submandibular duct was reached externally and exposed by separating the two digastricus muscles from one another and then penetrating the mylohyoid muscle. The ducts were cannulated with a fine polyethylene tube filled with distilled water and secured with two ligatures. Only one gland of each type was examined except for those rats exposed to chronic denervation, where also the contralateral, non-operated, gland was cannulated. By ligating the tube to the submandibular duct, and moreover cutting the duct just distal to the insertion of the tube as a further precaution, it was made sure that the submandibular gland was parasympathetically disconnected from the central nervous system. Evisceration was performed, under terminal anesthesia, by ligating the coeliac artery (which includes interruption of the hepatic arterial blood flow), the superior mesenteric artery, and the hepatic vein followed by the removal of the stomach, spleen, pancreas and the intestines. The liver, excluded from the circulation, was left in the body (III).

Blood pressure and glandular blood flow

A cannula was inserted into the femoral artery to enable continuous monitoring of the blood pressure. This was done by means of a pressure transducer connected to the cannula. A mean integrated response over 2 min or 5 min was calculated (I-V). To measure the blood flow through the submandibular gland the vein from the sublingual gland was ligated, as were some other tributary vessels (Greene, 1955). Blood flow was collected from the cannulated external jugular vein (IV). The animals were heparinized (1000 U/kg, intravenous). The venous drainage was collected into pre-weighed tubes in 2 min periods and estimated gravimetrically (the density of blood set at 1.0 g/ml). To preserve the blood volume, the collected blood was returned frequently to the animal via the cannulated femoral vein. The blood flow was expressed in µl per minute per 100 mg of gland.

Administration of test drugs

Drugs were given as an intravenous bolus dose. When the effect of either clozapine (I) or *N*-desmethylcozapine (II) was tested, the interval between subsecretory doses was usually 10-15 min. At secretory doses, the interval was prolonged until the secretion vanished or reached a steady level. When the effect of clozapine or *N*-desmethylclozapine on the methacholine- or nerve-evoked responses was tested, the interval between two doses was usually 15-20 min. Amisulpride and raclopride were given 10 min prior to the test sequence (IV).

To study the interaction between clozapine and *N*-desmethylclozapine, clozapine was administered initially (III). The peak secretory response to clozapine was usually reached within 10 min, after an additional 10 min *N*-desmethylclozapine was administered. To study the effect of amisulpride on the clozapine-induced secretion, amisulpride was administered 15 min subsequent to the administration of clozapine (III).

To test interactions between clozapine or *N*-desmethylclozapine and isoprenaline a fixed secretory dose of isoprenaline was administered (I, II).

Due to a drug half-life of about 1,5 h for clozapine and *N*-desmethylclozapine (Baldessarini et al., 1993, Sun and Lau, 2000), 1-2,5 h for olanzapine (Aravagiri et al., 1999, Kapur et al., 2003, Choi et al., 2007) and probably about 2 h for amisulpride, as judged by the pharmacokinetics of the closely related drug sultopride (Kobari et al., 1985), dose accumulations are likely to have occurred.

Reflex secretion

The parotid and submandibular ducts were cannulated as described above. Further, the tail vein was cannulated to provide a conduit for drug injections. The wounds were sutured and a xylocaine gel was applied to the area. About 2-3 h post surgery, a licking reflex could be elicited. At that time, the animals were drowsy and easy to handle. In the absence of atropine and adrenoceptor blockers, reflex secretion at a high flow rate was examined in the parotid gland. To study the reflex secretion at a low

flow rate the submandibular gland was chosen. Here, the parasympathetic cholinergic influence was eliminated by methylscopolamine, while at the same time avoiding central inhibition. Further, the submandibular gland was subjected to an acute parasympathetic preganglionic denervation to eliminate the participation of non-adrenergic, non-cholinergic transmission mechanisms (Ekström, 1998, Ekström, 2001). Twenty microliter of citric acid (5%) was applied, using a pipette, to the apex of the tongue every 30 sec in 5 or 8 min periods with intervals of 10 min (IV, V).

Stimulation of nerves

The parasympathetic auriculo-temporal nerve of the parotid gland was exposed where it emerges from the base of the skull, and the cervical sympathetic trunk was exposed in the neck. The peripheral end of either the auriculo-temporal nerve (postganglionic stimulation) or the ascending sympathetic nerve (preganglionic stimulation) was placed in a bipolar ring electrode. The nerves were electrically stimulated (6 V, 2 ms) using a Grass S48 stimulator and a Grass SIU 5A isolation unit (Grass Technologies Astro-Med, Inc., West Warwick, RI, USA). The parasympathetic innervation was usually stimulated in periods of 1 min to minimize a non-adrenergic, non-cholinergic influence (Ekström, 1998), while the sympathetic innervation was stimulated intermittently, in periods of 1 sec every 10^{th} second over 2 min to avoid impairment of the gland blood flow (Edwards, 1998).

Estimation of the secretory response

Since both clozapine and *N*-desmethylclozapine evoked secretion, a pre-existing flow of saliva was a complicating factor when the effect of these drugs on the methacholine-evoked or nerve-evoked secretion was to be assessed. The immediate preceding response was subtracted from the response subsequently evoked by the respective mode of stimulation. Comparisons were based on 5-min (methacholine), 2-min (sympathetic stimulation) and 1-min (parasympathetic stimulation) time periods.

Collection of saliva

The secreted saliva was collected in ice-chilled pre-weighed Eppendorf™ tubes or on filter papers and then re-weighed, to enable the flow to be estimated gravimetrically (assuming the density of saliva to be 1.0 g/ml). Saliva samples were expressed per gland or per 100 mg of gland. Samples to be analyzed for amylase activity were frozen and stored (-80°C) until they were assayed.

Assay of amylase

Saliva were assayed using an enzymatic colorimetric test (Boehringer GmbH, Mannheim,´ Germany) with 4-nitrophenylmalto-heptaosid (4NP-G7) as the substrate (Hägele et al., 1982). One unit (U) of the catalytic activity of amylase is defined as the hydrolysis of 1µmol of 4NP-G7/min per ml. The salivary amylase activity was expressed as the concentration (U/µl saliva, IV, V).

Chemicals

Atropine sulphate, 4-DAMP, isoprenaline hydrochloride, methacholine-chloride, methylscopol aminemethylnitrate, pirenzepine hydrochloride, propranolol, hydro-chloride, substance P and the substance P antagonist [D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}] were from Sigma Chemicals. The calcitonin gene-related peptide antagonist ratCGRP$_{8-37}$, *N*-desmethyl-clozapine, amisulpride and raclopride were from Tocris Bioscience. Phentol aminemesylate was from Novartis Pharma AG. Ketamine hydro-chloride was from Pfizer AB. Sodium pentobarbitone was from Apoteks-bolaget AB. Xylocaine gel was from Astra Läkemedel AB. Heparin sulphate was from Leo Pharma AB. Clozapine was a kind gift from Novartis Pharma AG.

Statistical analyses

Statistical significance of differences were calculated either with the Student`s *t*-test for paired or unpaired values, or by one-way analysis of variance (ANOVA), followed by Fisher`s protected least significant

difference or by repeated measures ANOVA, using Dunnetts's test or Bonferroni's test for selected pairs as post-test (GraphPad Prism). Comparisons were based on raw data or log values. Probabilities of <5% were considered significant. Values are the means ± standard error of the mean.

Results and Discussion

Clozapine-evoked secretion

A dose-dependent flow of saliva at a low rate was initiated from the "silent" non-secreting parotid and submandibular glands by the administration of clozapine (I). The submandibular glands responded to lower doses and with larger volumes as compared to the parotid glands. At secretory doses, saliva appeared 1-2 minutes upon the clozapine administration and, depending on dose, continued for 1-2 hours. The volume responses were enlarged in sensitized parotid and submandibular glands. Subsecretory doses of clozapine influenced the secretory cells, as revealed by the enlarged responses from the glands upon the administration of the β-adrenoceptor agonist isoprenaline (Figure 1). The clozapine-evoked secretion was unaffected by α- and β-adrenoceptor blockade but was completely abolished by atropine and completely or almost completely abolished by the muscarinic M_1 preferential receptor blocker, pirenzepine in the dose range 0.15-0.30 mg/kg, I.V. The secretory effect of clozapine was most probable due to a direct action on the muscarinic receptors of the secretory cells, since it was also demonstrated in glands disconnected from the central nervous system and in glands after the degeneration of the postganglionic parasympathetic and sympathetic nerves of the glands.

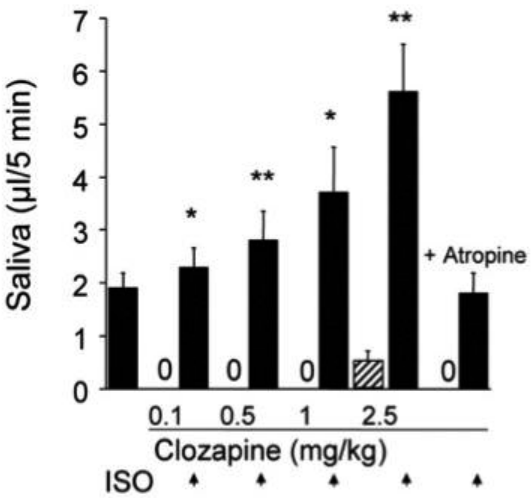
Clozapine-inhibited secretion

Clozapine exerted strong inhibitory actions, already at subsecretory doses (I). The methacholine-evoked response of the parotid and submandibular glands, as well as the parasympathetic nerve-evoked response of the parotid gland was almost abolished (≤ 90%) by raising the clozapine dose (10 mg/kg, I.V.). Clozapine did not exert any general depressive action on the secretory cells: the secretory response elicited by the injection of substance P was not affected by clozapine; and by prolonging the stimulation period of the parasympathetic nerve, after atropine

administration subsequent to the clozapine treatment, a non-adrenergic, non-cholinergic flow of saliva was recorded.

Moreover, the sympathetic-evoked flow from the submandibular gland was only reduced by 65% at the most by clozapine (3 mg/kg and 10 mg/kg). Administration of the α-adrenoceptor antagonist phentolamine caused no further reduction, while the β-adrenoceptor antagonist propranolol almost abolished the response. The lack of effect of the α-adrenoceptor antagonist phentolamine on the sympathetic response in the presence of clozapine, to be compared with a 75% reduction of the sympathetic response in its absence, suggests that the α-adrenoceptors were already blocked by clozapine. The persistent β-adrenoceptor sensitive response to the sympathetic stimulation and the absence of any reduction in the isoprenaline-evoked secretory response (in fact the responses increased, see above) in the presence of clozapine, showed clozapine to lack an antagonistic effect on $\beta_{(1)}$-adrenoceptors.

Figure 1



Synergistic interactions in submandibular glands of three rats. Mean response (± S.E.M) to fixed dose of ■ isoprenaline (2 µg/kg I.V) in a background of ▨ clozapine in increasing doses (0-2.5 mg/kg I.V) Adapted from paper I.

Reflex secretion

The citric acid-elicited high rate of reflex secretion from the parotid gland was markedly reduced by clozapine, already at subsecretory doses, by as much as 85% at a dose of 3 mg/kg (IV). Though, the action of clozapine is likely to be explained by its antagonistic action on the muscarinic M_3 receptors and the α_1-adrenergic receptors at the peripheral gland level, a central inhibition, in addition, cannot be ruled out.

N-desmethylclozapine-evoked secretion

The general pattern was the same for *N*-desmethylclozapine as for its parent compound, clozapine (II). The onset of secretion from the two types of glands was slow and the continuous flow of saliva was not reduced by the administration of α- and β-receptor blockers. The responses were magnified following chronic pre- or postganglionic denervation, a synergistic interaction with isoprenaline was demonstrated and further, the *N*-desmethylclozapine-induced salivary flow was abolished by atropine or pirenzepine (0.05-0.10 mg/kg). However, in contrast to clozapine, *N*-desmethylclozapine evoked secretion at lower threshold doses and caused the secretion of larger volumes.

N-desmethylclozapine-inhibited secretion

Like clozapine, *N*-desmethylclozapine reduced the methacholine- and nerve-evoked responses (II). At a dose of 10 mg/kg of *N*-desmethyl-clozapine, the methacholine-evoked response from the two glands was reduced by 40-60% and the parotid response to parasympathetic stimulation by 90% (1 Hz) and 70% (10 Hz). Evidently, the antagonistic efficacy was less for *N*-desmethylclozapine than for clozapine, see above. At the low frequency of parasympathetic stimulation, pirenzepine (0.1-0.2 mg/kg) abolished the response, while at the high frequency; it halved the response, which then was abolished by atropine. *N*-desmethylclozapine (3 mg/kg and 10 mg/kg) reduced the sympathetically nerve-evoked submandibular response by 70%, i.e. to about the same plateau level as achieved by clozapine. Also here, phentolamine was without effect on the persisting response, while it was abolished by propranolol.

Muscarinic M_1 and M_3 receptors

Functional studies show the rat submandibular gland to secrete to cholinergic stimulus by muscarinic M_1 and M_3 receptors. In the parotid gland, the secretory response is attributed to M_3 receptors, while a M_1 receptor contribution is questioned (Abrams et al., 2006, Ryberg et al., 2007, Tobin et al., 2009). However, the two types of muscarinic receptors have been localized, by immunohistochemistry, to the acinar cells of not only the submandibular gland but also of the parotid gland (Ryberg et al., 2007). In the present *Thesis* we found a secretory role also for the muscarinic M_1 receptor type in the parotid gland. A marked inhibitory effect (by 80-55%) on the methacholine-evoked volume response was demonstrated in innervated as well as in chronically denervated glands to pirenzepine in low doses (0.06-0.11 mg/kg, i.v) (II). Thus at dose levels where the antagonist is thought to display a selectivity for the muscarinic M_1 receptor (Tobin et al., 2002, Tobin et al., 2006). The clozapine- and *N*-desmethylclozapine-evoked secretion from the parotid and submandibular glands was also abolished at or close to these dose levels of pirenzepine. The residual response was ascribed to the muscarinic M_3 receptors, since it disappeared completely following the administration of 4-DAMP.

Our finding of functional muscarinic M_1 receptors in the serous parotid gland provides no support for the idea that the M_1 receptors is particularly engaged in the production of high-viscosity mucin-rich saliva (Watson and Culp, 1994, Abrams et al., 2006). Thus, the present finding is in agreement with the previous investigation on salivary glands of the ferret, showing no preponderance for M_1 receptors in the mucin-producing molar, sublingual and zygomatic glands compared to the serous parotid and sero-mucous submandibular glands (Khosravani et al., 2007).

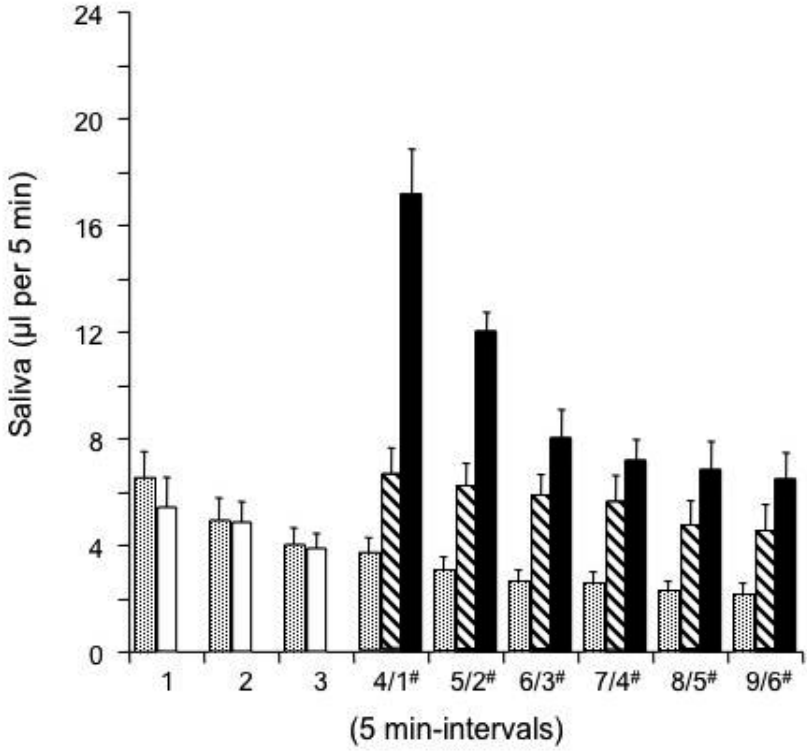
Combined action of clozapine and *N*-desmethylclozapine

Since clozapine both in humans and in rats is continuously metabolized by the activity of cytochrome P450 enzymes, localized to the liver and the intestines, it may be expected that not only clozapine but also its

metabolite *N*-desmethylclozapine contribute to the clozapine-induced secretion (Baldessarini et al., 1993, Eiermann et al., 1997). A high ratio of *N*-desmethylclozapine to clozapine in the blood might lessen the inhibitory effect on reflex secretion and increase its excitatory effect on the secretion. On the other hand, the weaker receptor agonist, clozapine, may occupy place for the stronger receptor agonist, *N*-desmethyl-clozapine, thereby reducing or abolishing an additive or synergistic effect of the two drugs on the secretory response. To study the interaction, the experiments were carried out on sensitized submandibular glands by preganglionic parasympathetic denervation in advance (III). Thereby, relatively strong secretory responses were obtained to clozapine and its metabolite, while at the same time the rat was exposed to relative low doses of the two drugs minimizing general systemic effects. In humans, the blood levels of clozapine and *N*-desmethylclozapine display considerable interindividual variations depending on a number of factors including age, gender, ethnicity, drug interactions and smoking. The average *N*-desmethylclozapine/clozapine ratios vary between different series of studies, 0.45-0.59 (Schaber et al., 1998, Guitton et al., 1999, Spina et al., 2000, Lee et al., 2009) and 0.75-0.90 (Bondesson and Lindström, 1988, Volpicelli et al., 1993). In rats, the corresponding ratio is 0.6 (Baldessarini et al., 1993).

Experiments on non-eviscerated and eviscerated animals (to prevent the breakdown of clozapine to *N*-desmetylclozapine), exposed to a wide range of clozapine/*N*-desmethylclozapine ratios (from 0.1 to 3), showed the combined action of the two drugs neither to attain a synergistic interaction nor a full additive effect (Figure 2).

Figure 2



Mean secretory responses (± S.E.M) in response to combined administration of clozapine and *N*-desmethylclozapine in the sensitized submandibular gland subjected to preganglionic parasympathetic denervation in advance, in the eviscerated rat. Saliva was collected over 5-min periods. In one group of six rats just ⁞⁞ clozapine (3 mg/kg, I.V.) was given. In another group of five rats just ■ *N*-desmethylclozapine (2 mg/kg, I.V.) was given; note that # indicates the responses of that group and that 1# represents the initial 5 min-period. In yet another group of eight rats □ clozapine (3 mg/kg, I.V.) was first given followed 15 min later by ▨ *N*-desmethylclozapine (2 mg/kg, I.V.). The experiments were performed in the presence of α- and β-adrenoceptor antagonists. Adapted from paper III.

Effects on the blood pressure by clozapine and *N*-desmethylclozapine

In the absence of any autonomic receptor antagonists, clozapine and *N*-desmethylclozapine, given separately, lowered the mean blood pressure to about the same level, from about 120 mm Hg to 70-80 mm Hg (I, II). The pressure fall is ascribed to loss of the sympathetic vasoconstrictor tone mediated by α_1-adrenergic receptors (Lameh et al., 2007). A pressure

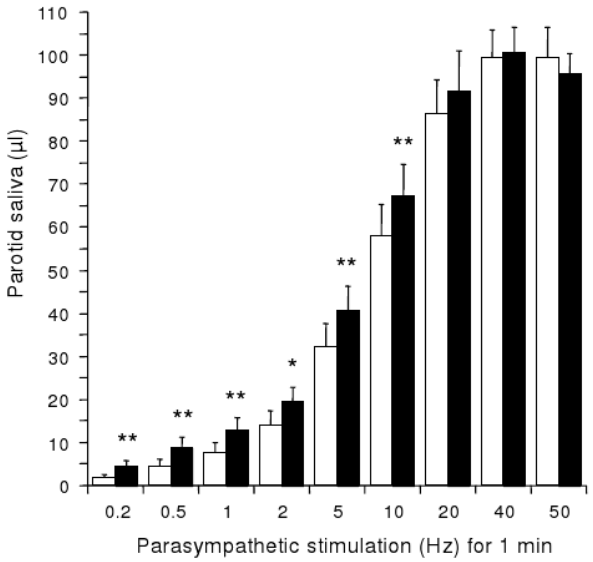
fall of the present magnitude is unlikely to affect the fluid secretion currently under study (Lung, 1990). Moreover, ongoing studies of ours showed clozapine, at a dose of 3 mg/kg, administered to the α- and β-adrenergic receptor antagonist-pretreated rat, just to induce a transient fall (by about 15 mm Hg) within the initial 2 min (Ekström J, Godoy T, Loy F and Riva A). Importantly, this dose of clozapine did not affect the blood flow through the submandibular gland over time and further, in response to the intravenous administration of vasoactive intestinal peptide a vasodilator response was still evoked of the same magnitude as in the absence of clozapine.

Effects of amisulpride

Based on a "nocturnal hypersalivation rating scale" amisulpride was reported to reduce the clozapine-induced sialorrhea in about 75% of the schizophrenic patients (Kreinin et al., 2010). No support for an inhibitory effect of amisulpride was found in the present experimental study, when amisulpride in increasing doses was intravenously administered during on-going clozapine-induced secretion from sensitized submandibular glands (III). On the assumption that the beneficial effect of amisulpride in the clinical situation depends on an attenuation of the nervous drive on the salivary glands rather than on a direct interference with the stimulatory action of clozapine, a series of experiments were performed (IV). However, neither in these experiments could an inhibitory action of amisulpride be demonstrated. On the contrary, amisulpride, causing no overt fluid secretion (or amylase release) on its own, enhanced the secretory responses to the electrical stimulation of the parasympathetic and sympathetic innervations (by 20-100 %) (Figure 3), and further to the administration of methacholine, bethanechol, isoprenaline and substance P (by 35-150 %). Moreover, the enhancing effect to agonists was still exerted in glands subjected to chronic parasympathetic and sympathetic denervation. No support for a central inhibition of amisulpride could be demonstrated. While the reflexly elicited secretion, at a high (maximal) flow rate was unaffected by amisulpride, the reflex secretion at a low flow rate, depending on an intact sympathetic innervation, was enhanced. Evidently, the secretory enhancing effect of amisulpride was not

associated with an action on the dopamine D_2/D_3 receptors, since raclopride, an antagonist to this receptor type was without any potentiating effect. Administration of amisulpride was without effect on the submandibular glandular blood flow, thus ruling out circulatory events *per se* as explanations to the enhancing secretory effect of the drug.

Figure 3



Mean secretory responses (+ S.E.M) from five rats to a series of stimulation frequencies applied to the parasympathetic auriculo-temporal nerve before □ the administration of amisulpride (15 mg/kg, I.V.) and after ■ in the presence of α - and β-adrenergic receptor blockade. Adapted from paper IV.

Effects of olanzapine

Olanzapine reduced the reflexly elicited secretion to citric acid (V). By increasing the dose of olanzapine, a long-lasting excitatory secretory effect on non-adrenergic, non-cholinergic receptors of the parotid and submandibular glands was unexpectedly added to the inhibitory secretory effect, already at hand, on the muscarinic receptors. The olanzapine-induced secretion occurred in the presence of atropine and α- and β-adrenergic receptor antagonists as well as after the degeneration of the postganglionic parasympathetic and sympathetic innervation. A number of non-traditional transmitters are known to act on the rat salivary glands

(see Introduction). Some of these evoke, when administered intravenously, no or just a small flow of saliva like calcitonin gene-related peptide or vasoactive intestinal peptide, while others evoke a large flow of saliva like the tachykinins, among them substance P (Ekström, 1999b). In the presence of subsecretory doses of olanzapine, the substance P-evoked response of the glands was not diminished. In the presence of ongoing olanzapine-induced secretion, the substance P-evoked response was largely magnified. From the working-hypothesis of an action of olanzapine on substance P receptors of the two types of glands, a substance P receptor antagonist was tested and found to reduce the olanzapine-induced secretion by 20-60% depending on flow-rate and dose of the antagonist. The inhibitory effect of the substance P antagonist on the olanzapine-induced secretion favors the engagement of substance P receptors in the olanzapine-evoked response. Moreover the amylase concentration in the olanzapine-evoked saliva was in the same range as the saliva evoked by substance P. However the difference in the magnitude of the secretory response of olanzapine and substance P between the two types of glands indicates that the two drugs do not exert identical actions. While substance P evokes larger volume responses from the submandibular glands than from the parotid glands, olanzapine evokes larger responses from the parotid gland than from the submandibular glands. Moreover, whereas the response to the administration of substance P was prompt, the response to olanzapine was delayed. Calcitonin gene-related peptide is known to potentiate the substance P-evoked secretory response (Ekström et al., 1988). However, no evidence for a complementary role for a calcitonin gene-related peptide receptor-mediated effect was found in the olanzapine-evoked secretion.

General Discussion

The four drugs currently under study were found to stimulate the secretion of saliva in our experimental animal model. However, the mechanisms behind their secretory actions varied. Clozapine and its metabolite *N*-desmethylclozapine evoked secretion by muscarinic M_1 receptors. Olanzapine evoked secretion by non-traditional receptors, probably involving substance P receptors of the tachykinin-family. Amisulpride evoked no secretion on its own. Nevertheless, it enhanced the secretory response evoked by a number of autonomimetics as well as by parasympathetic and sympathetic nervous activity.

Analytic pharmacology and surgery made it likely that the low-graded continuous flow of saliva evoked by clozapine and *N*-desmethylclozapine was due to a direct action on the muscarinic M_1 receptors of the glands independent of central nervous mechanisms, prejunctional neuronal events or circulating catecholamines. The fact that clozapine exerted its secretory effect in a situation where its degradation to *N*-desmethylclozapine was prevented underlines the secretory role of clozapine *per se*. A combination of the two drugs showed that both the ratio of *N*-desmethylclozapine/clozapine and the amount of clozapine administered were of importance in allowing *N*-desmethylclozapine to contribute to the volume of saliva secreted. Direct comparisons between the doses applied of clozapine in humans and those administered intravenously to the rat should be made with caution. In rodents, the half-live of antipsychotics is several fold faster than in humans, by 3 to 10 times with respect to clozapine. Applied as a single dose, clozapine in the range of 5 to 15 mg/kg are thought to reach clinically comparable doses as judged by the *in vivo* dopamine D_2 receptor occupancy (Kapur et al., 2003). With the aim to achieve a *N*-desmethylclozapine/clozapine ratio in the blood of the same range as that occurring in humans, and using a dose of clozapine comparable to that used in the clinical situation, our findings suggest that the contribution by *N*-desmethylclozapine to the volume response is, at the most, partly additive. The weaker agonist clozapine prevents the stronger agonist *N*-desmethylclozapine from

acting on the muscarinic M_1 receptor. Muscarinic M_1 receptors are not the only ones available for stimulation in the presence of clozapine and *N*-desmethylclozapine. A sympathetic secretion via the $\beta_{(1)}$-adrenergic receptors could still be evoked and further, a secretion via non-adrenergic, non-cholinergic receptors could be elicited as shown by the administration of substance P and by prolonged stimulation of the parasympathetic nerve. Not only did the $\beta_{(1)}$-adrenergic receptors by themselves, upon activation, contribute to the volume response but they did also mediate a synergistic interaction with clozapine and *N*-desmethylclozapine resulting in much larger volumes being secreted. Recent unpublished studies of ours show the parasympathetic transmitter vasoactive intestinal peptide, upon administration, also to enlarge the clozapine response. The synergism may be attributed to the intracellular interaction between inositol triphosphate/Ca^{2+} and cyclic AMP (Larsson et al., 1990, Hirono et al., 1998, Tanimura et al., 1999).

In certain experiments, the phenomenon of sensitization was made use of. The responses to both clozapine and *N*-desmethylclozapine were found to be enlarged in chronic preganglionic parasympathetic denervated submandibular glands and chronically postganglionically parasympathetic and sympathetic denervated parotid glands. Salivary glands are classical neurobiological model organs in exploring the phenomenon of postjunctional supersensitivity (Emmelin, 1965, Ekström, 1999a). The sensitization develops gradually and reaches its maximum within 2-3 weeks. The sensitivity of the glands to secretagogues is regulated by the transmitter bombardment of the receptors. A receptor stimulation below the "normal" level leads to supersensitivity, whereas stimulation above that level leads to subsensitivity. Thus, the salivary glands of rats kept on a liquid diet (giving rise to a low reflex stimulation) show a higher degree of sensitivity to secretagogues than those glands of rats kept on a chewing demanding diet (giving rise to a high reflex stimulation, Ekström and Templeton, 1977). The sensitization achieved by surgical or pharmacological means is higher after postganglionic denervation (depriving the gland not only of the amount of transmitter continuously released from the nerve endings but also of that amount released upon salivary reflexes) than after preganglionic denervation (only abolishing the transmitter amount

released in response to the reflex activity) illustrating "Cannon´s law of denervation" (Emmelin, 1965). In salivary glands, as in some other autonomically innervated structures, the development of supersensitivity is thought to reflect changes beyond the receptor level (Ekström, 1999a). The degree of sensitivity is not correlated to changes in the density of receptors but rather to the disuse of the various intracellular pathways. Postjunctional supersensitivity is usually thought of as an unspecific phenomenon, though agonist-specific patterns can be demonstrated (Ekström, 1999a). In the clinical situation, chronic blockade of the muscarinic M_3 receptors and the α_1-adrenergic receptors by clozapine and *N*-desmethylclozapine, creating a "partial" pharmacological de-nervation of the gland, may hypothetically increase the sensitivity of the secretory cells to stimulants of non-blocked receptors. On the other hand there is also the possibility that the continuous stimulation of the muscarinic M_1-receptor counteracts the development of sensitization.

The exploration of the various functions of the five muscarinic receptor subtypes in salivary glandular activities is hampered by the fact that the selectivity of the pharmacological tools is relative rather than absolute. The similarity in ligand binding sites across the M_1-M_5 subtypes of receptors is thought to be the principal reason for difficulties in identifying ligands displaying subtype-selectivity (Jöhren and Höltje, 2002). Pirenzepine, currently used, is usually regarded as a M_1-selective antagonist but may, to a less degree, exert an antagonistic effect also on M_4 receptors (Caulfield, 1993, Caulfield and Birdsall, 1998, Eglen and Nahorski, 2000). 4-DAMP, also currently used, is an antagonist to $M_1/M_3/M_5$ receptors but not to M_2/M_4 receptors (Caulfield, 1993). Though, immunoblotting and immunohistochemistry show the presence of all five subtypes of muscarinic receptors, at various locations, in salivary glands, functional studies indicate M_3 and M_1 receptors in mediating salivary *fluid* secretion (Ryberg et al., 2007), a view supported by the current findings. Early studies using cell lines transfected with human muscarinic M_1-M_5 receptors and exposed to clozapine, focused on an agonistic action of clozapine on M_4 receptors as indicated by an inhibition on the cyclic AMP formation (Zorn et al., 1994, Zeng et al., 1997). An agonistic action of clozapine on muscarinic M_4 receptors as explanation for the clozapine "hypersalivation" was suggested (Zorn et

al., 1994). Further *in vitro* cell-based assays showed clozapine to be a weak partial agonist at M_1 receptors, a more efficacious agonist at M_2 and M_4 receptors, and to lack agonistic activity at M_3 and M_5 receptors. *N*-desmethylclozapine, tested in the same *in vitro* system, showed increased agonist efficacy at M_1, M_4 and M_5 receptors compared with clozapine (Weiner et al., 2004). That, the picture is not always clear-cut may be illustrated by the report of Thomas and co-workers (Thomas et al., 2010), where *N*-desmethylclozapine is found to be an antagonist at the native human M_1 receptor, but a partial agonist at the human recombinant M_1 receptor. Both clozapine and *N*-desmethylclozapine are thought to modulate M_1 receptor activity via an allosteric site that partially overlaps with the orthosteric binding site for acetylcholine (Sur et al., 2003, Eglen, 2012).

Although, both the salivary effect of clozapine and the attempt to reduce it by pirenzepine have been associated with the muscarinic M_4 receptor in the past (Zorn et al., 1994, Praharaj et al., 2006, Sockalingam et al., 2007) a direct role for postjunctional M_4 receptors in the clozapine-induced *fluid* secretion seems less likely. The immunohistochemistry locates this type of receptor to the vicinity of acini rather than to the acinar cell membrane (Ryberg et al., 2007). Moreover, in functional studies no support was gained for a postjunctional secretory role for the M_4 receptor subtype (Tobin et al., 2006). However, functional studies suggest that these receptors are located prejunctionally and upon stimulation, exert an inhibitory action on transmitter release (Tobin et al., 2009).

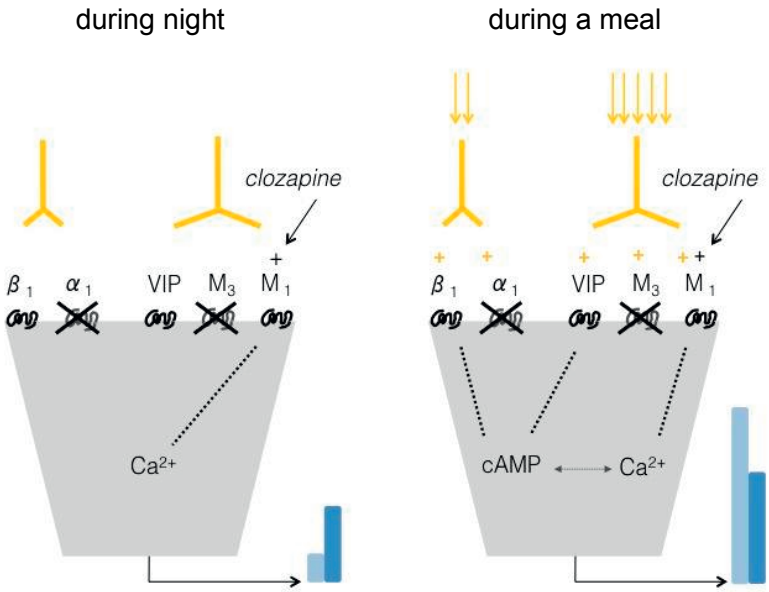
In human labial glands, immunohistochemistry found the M_1 and M_3 receptors evenly distributed, while the acinar cells lacked M_2 and M_4 (Ryberg et al., 2007). Turning to the human parotid and submandibular glands, the acinar serous cells show immunoreactive staining for the M_1 receptor (Isola, R., University of Cagliari, personal communication).

The muscarinic M_1 receptors are not only located to the secretory cells, as discussed above, but also to the presynaptic site where they, upon stimulation, facilitate the release of transmitters, including non-adrenergic, non-cholinergic ones (Tobin, 2002, Tobin et al., 2009). Thus, it may be worthwhile to consider the facilitatory action of the presynaptic

muscarinic M_1 receptors when searching for complimentary explanations to the clozapine-induced sialorrhea.

A tentative explanation to the reported mixed secretory actions of clozapine in the clinic seems to emerge (Figure 4). Looking at the extremes: during the night, the clozapine/*N*-desmethylclozapine-induced secretion, by the stimulation of the postjunctional muscarinic M_1 receptors, adds to the spontaneous secretion causing the wet pillow: during a meal, the secretory response is likely to be reduced, since the reflexly released acetylcholine and noradrenaline are prevented by clozapine/*N*-desmethylclozapine from acting on the muscarinic M_3 receptors and α_1- adrenergic receptors of the secretory cells.

Figure 4



A tentative explanation to the reported mixed action of clozapine. VIP, vasoactive intestinal peptide, symbolizing the non-adrenergic, non-cholinergic transmitters. Expected salivary response in the absence of clozapine, and in the presence of clozapine.

Upon contraction of the myoepithelial cells the ductal pressure increases allowing the gland to respond promptly (Thulin, 1975, Garrett and Emmelin, 1979). Myoepithelial cells of the parotid and submandibular glands of the rat are activated by muscarinic agonists (Thulin, 1974, Thulin, 1975). Immunohistochemistry suggests that the myoepithelial cells may be supplied with muscarinic M_1 receptors (Ryberg et al., 2007). The slow onset in the appearance of saliva in response to the administration of clozapine or *N*-desmethylclozapine gives, however, no support for the participation of myoepithelial cells in the evoked response.

As previously mentioned, when this doctoral work began, no studies were on record objectively measuring the amount of saliva secreted in response to clozapine in humans, and further no studies included pre-administration secretory baseline values. However, during the course of the current studies, Praharaj and co-workers (2010) reported patients under the treatment with clozapine to show elevated salivary flow rates (by 40-70%) during day-time at rest, 1-4 weeks after the start of treatment. Here, saliva was collected on cotton balls placed under the tongue over 2 min-periods.

Introductory, several categories of drugs as well as surgery were presented as treatment for the clozapine-induced sialorrhea. However, from an oral health point of view salivary secretion in patients under anti-psychotic treatment should in general be regarded as a beneficial side-effect. Nevertheless if interventions are found necessary, aiming at reducing the salivary flow rate, they ought to be based on scientific ground. Though there is the possibility that the chronic clozapine therapy may induce adaptative changes in humans, the present *Thesis* may provide rationale for the treatment of the clozapine-induced sialorrhea.

A β-adrenoceptor blocker preferably of the β_1-type might be suitable to abolish not only the β_1 adrenergic receptor mediated secretion *per se* but also the potentiating effect of this receptor type on the clozapine muscarinic M_1 receptor-mediated secretion.

A more immediate alternative would be to use a selective muscarinic M_1-receptor antagonist. In the present *Thesis*, the preferential muscarinic M_1-receptor antagonist pirenzepine was used. This drug is marketed in some countries to reduce the secretion of gastric acid. In fact, the therapeutic effect of pirenzepine on clozapine-induced sialorrhea has been put to the test but with inconclusive outcomes. However, once again the studies either lack the direct objective measurement of the salivary flow rate or they lack appropriate controls. Fritze and Elliger (1995) concluded at bedside that the formely wet pillow stayed dry in a large number of patients after pirenzepine and in a later study, less hypersalivation to clozapine was reported by patients after pirenzepine, but no actual assessments were performed by the authors (Schneider et al., 2004). However, in the study of Bai and coworkers (2001) it was concluded that pirenzepine had no significant therapeutic effect on the clozapine-induced salivation. Though this was a double blind, placebo-controlled, crossover trial, the saliva secreted during the night was estimated from the diameter of the saliva-wetted surface of the pillow in the morning. Interestingly, in line with the interpretation of the present data, Bai and co-workers discussed in their study that the net effect of clozapine on salivation depended on which muscarinic receptor type, M_3 or M_4 (believed to mediate the secretion) did get the upper hand. Since, no support for an inhibitory pirenzepine action on the clozapine-sialorrhea was gained, Bai and co-workers referred the sialorrhea to deglutition disturbances rather than to neuroglandular events.

Amisulpride *per se* evoked no secretion from the glands and exerted no inhibitory action on the secretory cells. The effect was the contrary. Currently, there is no explanation at hand to the enhancing effect of amisulpride on the salivary volume response evoked by the para-sympathetic and sympathetic innervations or by agonists, involving the action on muscarinic, α- adrenergic, β-adrenergic and substance P-ergic receptors and thus, including the intracellular pathways inositol triphosphate/Ca^{2+} and cyclic AMP. It was possible to exclude central nervous sites as well as peripheral nervous sites of actions of amisulpride. The effect of amisulpride could not be attributed to circulating catecholamines, neither to any anti-cholinesterase activity (Fontaine and

Reuse, 1980, Nasello et al., 2003), prolonging the action of muscarinic agonists, nor to its blockade of dopamine D_2/D_3 receptors or to an effect on the glandular blood flow. Recent observations of ours show pieces of human submandibular glands exposed to amisulpride *in vitro* (Loy et al., 2011) and parotid and submandibular glands exposed to amisulpride *in vivo* to display ultrastructural changes associated with secretory activity and, as shown in the experimental gland of the current *Thesis*, without causing the secretion of fluid or the output of amylase *per se*. Not only do these recent observations underline an action of amisulpride at the gland level but imply also that amisulpride may put the secretory machinery in a state of readiness.

The potentiating effect of amisulpride on the secretory responses evoked by nerves and various agonists makes amisulpride or a related molecule to a potential drug for the treatment of dry mouth. Applied locally on the oral mucosa, it may like the cholinesterase inhibitor physostigmine (Ekström and Helander, 2002, Khosravani et al., 2009), diffuse through the epithelium to enhance the parasympathetic tone on the underlying minor glands and thereby, increase the rate of the mucin-rich secretion from this type of gland.

In contrast to clozapine and *N*-desmethylclozapine, olanzapine evoked secretion of saliva in the presence of atropine. Olanzapine initiated a conspicuously rich flow of saliva, probably by a direct action, as shown in denervation experiments, engaging non-adrenergic, non-cholinergic receptors and, at least partly, involving tachykinin-receptors of substance P-type. Nevertheless, its secretory effect on human glands may be questioned. In humans, substance P tested *in vitro* on submandibular gland tissue evokes no potassium release to indicate a fluid response (Larsson et al., 1986). In the rat salivary glands, nerve fibres containing either substance P or calcitonin gene-related peptide are found close to the acinar cells. In addition, nerve fibres containing substance P co-localized with calcitonin gene-related peptide, thought to be sensoric, occur close to vessels and ducts (Ekström et al., 1988). In rat salivary glands, calcitonin gene-related peptide evokes no fluid secretion but it enhances the substance P-evoked response (Ekström et al., 1988, Iwabuchi and Kimura, 1998). Moreover, in several species, including the

rat, calcitonin gene- related peptide causes vasodilatation (Tobin et al., 1991, Salo et al., 1994). It does also potentiate the substance P-evoked protein extravasation in the glands (Asztely et al., 1998). However, no support for an action of olanzapine on calcitonin gene-related peptide receptors was presently found. In human salivary glands, the acinar cells lack nerves containing substance P or calcitonin gene-related peptide but, like the rat salivary glands, vessels and ducts are supplied with substance P/calicitonin gene-related peptide containing nerve fibres, probably of sensoric nature (Hauser-Kronberger et al., 1992, Heym et al., 1994). A secretory role for olanzapine in human glands via non-adrenergic, non-chonergic receptors other than the substance P type cannot be excluded It might perhaps be worth considering a role for olanzapine in inducing or maintaining inflammation in salivary glands and elsewhere by its action on substance P receptors and by its potential positive interaction with calcitonin gene-related receptor-mediated effects. Interestingly, substance P may not only contribute to the inflammatory response by its direct action on the blood vessels causing vasodilation, protein extravasation and oedema but also indirectly on substance P receptors of the mast cells inducing degranulation (Zhang et al., 2012). In rats, chronic olanzapine treatment causes a low-grade inflammatory state in the adipose tissue (Victoriano et al., 2010). In humans, stimulation of substance P receptors is associated with inflammation in the bowel and in the urinary bladder (Gross and Pothoulakis, 2007, Arms and Vizzard, 2011). Moreover, it may be wondered whether the weight-gain in patients under olanzapine treatment is associated with an action of the drug on tachykinin receptors (Green, 1999, Karagiannides and Pothoulakis, 2009). Though, the results of our experimental work provide functional support for an action of olanzapine on substance P receptors, it should be pointed out that the only binding assay on record does not favor such an assumption. In the astrocytoma cell line U373MG (Heuillet et al., 1993), olanzapine was reported to have a negligible affinity to substance P receptors (Theisen et al., 2007). Evidently, the discrepancy calls for further studies.

As judged by the work of Kapur and coworkers (2003), the dose of olanzapine evoking secretion presently used is in the high range in comparison with the clinical situation. At a potency lower, the dose

becomes comparably to that applied to humans and here, olanzapine markedly lowered the methacholine-evoked response as well as the reflexly elicited response.

Though, the antimuscarinic effect of olanzapine was similar to that of clozapine, complains of dry mouth is more frequent among patients under olanzapine treatment than after clozapine treatment (Tollefson et al., 2001, Duggan et al., 2003). The reason for the difference might be the clozapine-induced muscarine M_1-mediated secretion.

Eventually, it should be pointed out that one ought to be cautious in extrapolating preclinical findings to the clinical situation. For instance, apart from differences in pharmacokinetics, route of administration and glandular receptor profile, the animals were acutely subjected to the drugs, whereas patients are under chronic treatment, possible inducing adaptative changes at central and glandular levels.

Future studies may focus on the clinic and the objective measurements of the secretory effect of the drugs currently under study. For instance, a role for β_1-adrenoceptor antagonists and also for pirenzepine in the treatment of clozapine-induced sialorrhea would be of interest to investigate. Moreover, the finding of a secretory potentiating effect of amisulpride may serve as a starting point for the development of a drug to treat dry mouth, a development that perhaps also may include *N*-desmethylclozapine.

Main Conclusions

Clozapine and its metabolite *N*-desmethylclozapine stimulated the parotid and submandibular glands to produce a continuous low-graded flow of saliva by their direct action on the muscarinic M_1 receptors of the glands. The two drugs exerted inhibitory actions on the M_3 receptors and on the $\alpha_{(1)}$-adrenergic receptors. They did, however, not reduce the secretion mediated by the $\beta_{(1)}$-adrenergic receptor or by the substance P-receptor. On the contrary, both clozapine and *N*-desmethylclozapine, even in subsecretory doses, interacted positively with the β-adrenergic receptor agonist isoprenaline, resulting in enlarged fluid responses to the administration of the β-receptor stimulant.

With respect to the muscarinic receptor subtypes, clozapine was a weaker agonist and a stronger antagonist than *N*-desmethylclozapine. When acting together in a situation, where the breakdown of clozapine was prevented, the weaker agonist attenuated the secretory response to the stronger agonist. Both the ratio *N*-desmethylclozapine/clozapine and the dose of clozapine were of importance for the magnitude of the volume response. An extrapolation to the clinical situation suggests that, at most, a partly additive secretory effect may occur.

Thus, the wet pillow and the drooling are explained by a continuous agonistic action on the muscarinic M_1-receptor by clozapine/*N*-desmethylclozapine. When the demands on the secretory activity of the glands increase, such as during a meal, the antagonistic action of clozapine/*N*-desmethylclozapine on the muscarinic M_3-receptor and the α_1-adrenergic receptor is likely to come to light and the patient under treatment will experience the diminished saliva production.

Amisulpride, introduced as a drug to stop the clozapine-induced sialorrhea, was without effect with respect to this phenomenon. The drug did, however, without causing any fluid secretion on its own, enhance the secretory response to reflex secretion (from a low flow rate) and to stimulation of the parasympathetic and sympathetic innervations and further, to the administration of drugs mobilizing either of the two

intracellular pathways, inositol triphosphate/Ca^{2+} and cyclic AMP. Denervation experiments made it likely that amisulpride exerted its action on the gland level. Currently, there is no explanation to the amplifying effect of amisulpride. The drug is of potential interest for the treatment of dry mouth.

Olanzapine-evoked a continuous flow of saliva over a period of time like clozapine and *N*-desmethylclozapine but in contrast to these drugs, the secretion was not blockable by muscarinic or adrenergic receptor antagonists. The non-adrenergic, non-cholinergic fluid secretion evoked by olanzapine was a peripheral effect partly engaging the tachykinin (substance P) receptors of the secretory cells. In humans, olanzapine is not likely to evoke salivary secretion, since the secretory cells in the human salivary glands lack a substance P-innervation. However, substance P-nerve fibres in the human salivary glands innervate vessels and ducts, and are part of the sensory innervation. An olanzapine treatment in humans may be a potential stimulus to an inflammatory response via substance P receptors, causing vasodilatation and the formation of oedema, in salivary glands as well as in other parts of the body.

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