# CHARACTERISATION OF DOPAMINE NEURONS OF THE MURINE VENTRAL TEGMENTAL AREA IN PRIMARY CULTURE AND ACUTE DISSOCIATIONS

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**Tiivistelmä**

Dopaminergiset hermosolut säätelevät liikunnallisia, endokriinisiä ja kognitiivisia toimintoja. Näiden hermosolujen rappeutumisen ja häiriintyneen säätelyn uskotaan vaikuttavan useiden solujen kehitykseen ja rappeutumiseen liittyvien neurologisten oireyhtymien, sekä tiettyjen psykiatristen ja geneettisten sairauksien syntyyn. Nisäkkäillä, dopamiinia valmistavat ja vapauttavat solut on jaettu erilisiin A16-A8 -pääryhmiin, sekä pienempiin vlPAG- ja DRN-alaryhmiin. Tämän työn tarkoitus oli tutkia näiden solujen levinneisyyttä ilmentymiskartan avulla, joka tehtiin nuorista ja vanhoista transgeenisen Th-EGFP -kannan hiiristä, jossa dopamiinisolut ovat tunnistettavissa visuaalisesti. Tulokset osoittavat, että 3 viikon ikäisillä hiirillä dopaminergisten hermosolujen levinneisyys on aikuisten hiirten kaltainen, jolloin olisi mahdollista tehdä kokeita myös nuoremmilla eläimillä.

Tutkielman pääpaino oli A10-pääryhmässä, jonka hermosolut ovat ventraalisella tegmententaalisella alueella (VTA). Näiden solujen hermorata muodostaa kohdealueineen mesokortikolimbisen järjestelmän, joka tunnetaan myös aivojen palkkioradan osana. Työn toinen tarkoitus oli verrata olemassa olevia primaarisia soluviljelymalleja sekä akuutteja mekaanisia eristyksiä VTA:n dopamiini hermosoluille. Tulokset osoittivat että hyväkuntoisten dopamiinisolujen saanto on suurempi soluviljelymallilla, varsinkin kun astrosyyttejä (jotka toimivat tukisoluina) rikastetaan kylmän liuospesun avulla ja hermosolut puhdistetaan ilman sentrifugointia ja viljelmille lisätään GDNF-kasvutekijää.

VTA:n dopamiinisolujen aktiivaatio lisää glutamaatti-välitteisen hermosäätelyn määrää pitkäaikaisesti (ns. pitkäaikaisvahvistuminen engl. long-term potentiation), mikä on hermostossa yleisesti otaksuttu perusta kognitiivisille tapahtumille, kuten muistamiselle ja oppimiselle. Tässä tutkimuksessa glutamaatista riippuvaisten ionikanavien vasteita tutkittiin sähköfysiologisin menetelmin. Mittausten tulokset muistuttivat AMPA-tyyppisten glutamaattireseptorien välittämiä virtauksia. Yhteenvetona voidaan todeta, että primaarinen astrosyyttien ja hermosolujen yhteisviljelmä on erinomainen *in vitro* -malli VTA:n fysiologian tutkimiseen ja huolimatta puuttuvasta tilastollisesta validoinnista, alustavat glutamaattimittaukset viittaavat siihen, että metodi pystytettiin onnistuneesti tämän työn aikana.

Avainsanat: dopamiini, ilmentymiskartta, primaarinen yhteisviljelmä, glutamaatti, mesokortikolimbinen järjestelmä, plastisuus, sähköfysiologia

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**Abstract**

Dopamine (DA) neurons play pivotal roles in the regulation of motor and endocrine functions, as well as affective and cognitive behaviours. The degeneration and dysregulation of these neurons has been linked to the pathophysiology of several neurodegenerative, neurodevelopmental and psychiatric diseases, as well as genetic disorders. In the mammalian brain, neurons that synthesise and release DA are distributed to distinct major A16-A8 nuclei and smaller subnuclei labelled vlPAG and DRN. The aim in this project was to investigate the distribution of these neurons by creating an expression map of perfused brain samples from young and adult animals derived from a transgenic Th-EGFP mouse line in which DA neurons were visually identified. The results indicated that 3-week-old animals show mature DA neuron distribution and thus present the possibility to conduct experiments with these young adolescent animals.

Emphasis of this project was on the DA neurons of the ventral tegmental area (VTA) with projections constituting the mesocorticolimbic system. This pathway is regarded as a critical regulator of reward and goal-directed behaviour. The second aim of the project was to compare pre-existing methods of primary co-culture and acute mechanical dissociations to set up an *in vitro* model for VTA DA neurons. The results showed that a higher yield of viable neurons is obtained with the primary culture particularly, when astrocytes (that are required for growth support) are enriched through cold wash and neurons are purified without centrifugation and the cultures are supplemented with glial derived neurotropic factor (GDNF).

Excitatory glutamatergic modulation of VTA is suggested to regulate important functions of synaptic plasticity, which have been associated with learning and memory. Here, currents mediated by the glutamate-gated ion channels were investigated by applying glutamate to patched neurons and recording the responses in whole-cell mode. The acquired traces resembled the currents mediated by AMPA-type glutamate receptors. In summary, primary astrocyte neuron co-culture combined with the Th-EGFP mouse strain is an excellent *in vitro* model for studying VTA physiology, and albeit statistical analysis remains to validate the appropriate reproducibility of the protocol, the initial results on glutamate responses suggest that such methodology was successfully set up during the course of this thesis.



Key words: dopamine, expression map, primary co-culture, glutamate, mesocorticolimbic system, plasticity, electrophysiology

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**Abbreviations**

| | |
|---|---|
| ADHD | Attention deficit hyperactivity disorder |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| ARC | Arcuate nucleus |
| ASD | Autism spectrum disorders |
| DA | Dopamine |
| DAPI | 4,6-diamidino-2-phenylindole |
| DRN | Dorsal raphe nucleus |
| FUDR | 5-Fluoro-2'-deoxyuridine |
| FXS | Fragile X syndrome |
| CAMKII | $Ca^{2+}$/calmodulin-dependent protein kinase II |
| cAMP | Cyclic adenosine monophosphate |
| CNS | Central nervous system |
| GABA | γ-Aminobutyric acid |
| GDNF | Glial derived neurotropic factor |
| ICD | Impulse control disorder |
| Kainate | 2-carboxy-3methyl-4-isopropenylpyrrolidine |
| L-DOPA | L-dihydroxy-phenylalanine |
| lPOA-rHA | Lateral preoptic–rostral hypothalamic area |
| LTD | Long-term depression |
| LTP | Long-term potentiation |
| NMDA | N-methyl-D-aspartate |
| OCD | Obsessive compulsive disorder |
| PDD | Pervasive developmental disorder |
| PFA | Paraformaldehyde |
| PFC | Prefrontal cortex |
| PBS | Phosphate-buffered saline |
| SUD | Substance abuse disorder |
| PPTg | Pedunculopontine tegmentum |
| Th-EGFP | Tyrosine hydroxylase-Enhanced Green Fluorescent Protein |
| TIDA | Tuberoinfundibular dopamine neurons |
| vlPAG | Ventrolateral periaqueductal grey |
| VTA | Ventral tegmental area |
| ZI | Zona incerta |

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# 1. Introduction

The brain is the most intricate, energetically active, and plastic organ in the body and its fundamental task is to communicate and process information. Neurons convey this information through the gap junctions of electrical synapses and through the neurotransmitter-filled vesicles of chemical synapses. Dopamine (DA) is a widely studied neurotransmitter because of the pivotal roles DA neurons have in the regulation of motor and endocrine functions, as well as affective and cognitive behaviours. Furthermore, the degeneration and dysregulation of these neurons have been linked to pathophysiology of several disorders which have major socioeconomic impact on our society: drug addiction and depression constitute the greatest health problems in industrialized countries and the worldwide incidence of schizophrenia remains constant at about 1% (Prakash & Wurst, 2006).

This thesis focused on the distribution of murine DA neurons, which are known to locate to stereotypic nuclei designated A16-A8 as well as few minor subnuclei. The emphasis was on the neurons located in the ventral tegmental area (VTA) of the midbrain – the critical regulator of reward and goal-directed behaviour. The neurons originating from this region project and receive input from various other brain regions and through several neurotransmitter systems. The attention was concentrated on the excitatory modulation suggested to regulate important functions of synaptic plasticity, which have been associated with learning and memory.

The methodology for studying the characteristics of DA neurons *in vitro* is relatively well-established in the literature (Fasano et al., 2008; Congar et al., 2002; Frank et al., 2008; Sulzer et al., 1998). Nevertheless, optimisation and validation require effort implemented by novel working conditions and environment. In this study, pre-existing models were set up and compared to discover the most efficient approach that would eventually be utilised for studying the physiology of VTA DA neurons and their glutamatergic regulation using whole-cell patch-clamp electrophysiology.

# 2. Review of the literature

## 2.1. The dopaminergic system

Dopamine, like all biogenic amines, is synthesised from the aromatic amino acid tyrosine that is first converted to L-dihydroxy-phenylalanine (L-DOPA) by the rate-limiting enzyme tyrosine hydroxylase (Th). L-DOPA is then transformed to DA by the L-aromatic amino acid decarboxylase. The neurons that synthesise and release dopamine as the neurotransmitter are by definition dopaminergic (Flames and Hobert, 2011). However, recent studies have suggested that some of the neurons are capable of co-releasing other neurotransmitters such as glutamate (Hnasko et al., 2010; Stuber et al., 2010) or GABA (Tritsch et al., 2012).

At the acceptor cells, the effect of DA is mediated by the G-protein-coupled receptors which are divided into two main groups based on their ligand affinity and specificity as well as effector coupling: $D_1$-like subtypes of $D_1$ and $D_5$, and $D_2$-like subtypes of $D_2$, $D_3$ and $D_4$. The $D_1$-like receptors are coupled to $G_\alpha$-protein and activate the cyclic adenosine monophosphate (cAMP) synthesising enzyme adenylate cyclase, whereas the $D_2$-like receptors are coupled to $G_i/G_o$-proteins and decrease the activity of the enzyme (Bratcher et al., 2005; Callier et al., 2003).

### 2.1.1. Diversity of DA populations

DA was first discovered by Carlson, Falck and Hillarp in 1962. Two years later, Dahlström and Fuxe (1964) divided the neurons based on their stereotypic anatomical locations. After the introduction of immunocytochemistry, the DA system could be investigated at a greater detail, yet the original nomenclature has been retained (Björklund and Dunnett, 2007). Today, 10 major DA nuclei are identified in mammals (Figure 1). Most rostral populations are found in the telencephalon, comprising the A16 group of olfactory bulb periglomerular interneurons and the A17 group of amacrine interneurons in the retina, neither of which possess dendrites (Prakash and Wurst, 2006; Turiault et al., 2007).

Another group of DA neurons comprising the A15–A11 nuclei occupy various parts of the diencephalon. The location of the A15 is controversial in literature as it is identified either in the preoptic nucleus (Molnar et al., 1994; Turiault et al., 2007), supraoptic nucleus (Prakash & Wurst, 2006a) or the nuclei is excluded or

determined absent (Albanese, et al., 1986; Qu et al., 2006). The adjacent A14 nucleus is located in the paraventricular hypothalamus and the largest nuclei A13 and A11, are located in the posterior hypothalamus and zona incerta (ZI) in the ventral thalamus, respectively (Prakash & Wurst, 2006). The remaining A12 nucleus includes the hypothalamic tuberoinfundibular DA neurons (TIDA) which originate in the arcuate nucleus (ARC) (Benskey et al., 2012; Phelps, 2004).

Neuron populations constituting the origin of the mesencephalic DA system are predominantly located in the ventral midbrain region: the A9 nucleus is located in the substantia nigra and the A10 nucleus, the major focus of this thesis, is located in ventral tegmental area. The most caudal nucleus is A8 which comprises the DA neurons of the retrorubral field (Björklund and Dunnett, 2007; Prakash and Wurst, 2006). In addition to the A16-A8 major nuclei, subpopulations of approximately 1000 DA neurons (in rat) have been identified in the dorsal raphe nucleus (DRN) and ventrolateral periaqueductal grey (vlPAG) regions. These are often referred to as the rostral-caudal extension of A10 (Dougalis et al., 2012). All of the listed DA neurons receive inputs from, and project to other parts of the brain, thus modifying different features of behaviour.

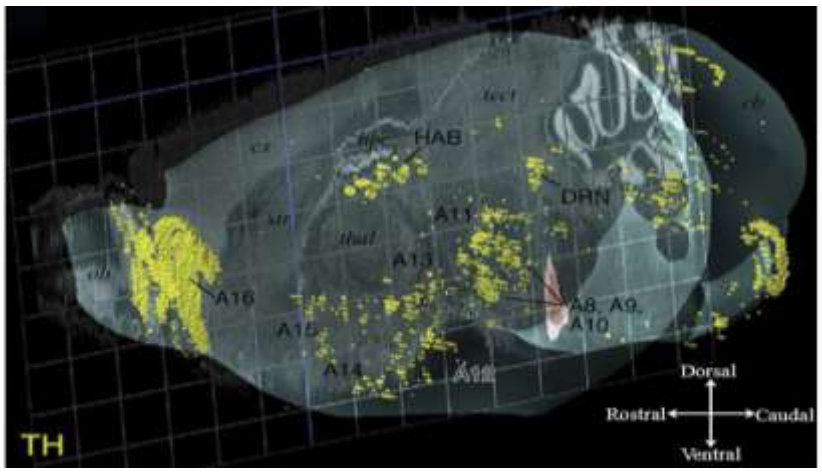


**Figure 1 Distribution of DA neuron cell groups in the adult brain marked by the expression of tyrosine hydroxylase expression (Th).** DA neurons are divided into 9 distinct groups ranging from olfactory bulbs (A16) to diencephalon (A15-A11) and mesencephalon (A9-A8). The anatomical orientation is defined by the coordinate system of adjacent head region: Rostral (nose), caudal (back of the head), dorsal (top of the head), ventral (neck). Abbreviations: cb (cerebellum); cx (cortex); hpc (hippocampus); ob (olfactory bulb); str (striatum); tect (tectum); thal (thalamus). Image adapted and modified from (Björklund and Dunnett, 2007).

### 2.1.2. Functions and dysfunctions of VTA DA neurons

As well as having stereotypic anatomical locations, all described DA nuclei

have characteristic projection areas (Prakash & Wurst, 2006). In the case of VTA (A10 nucleus), the projections are generally divided into two systems: The *mesolimbic* pathway comprises the projections to the nucleus accumbens, olfactory tubercle, septum, amygdala and hippocampus whereas the subset known as the *mesocortical* pathway comprises the projections to the medial prefrontal cortex, cingulate and perirhinal cortex. Collectively, these are often referred to as the *mesocorticolimbic* system (Wise 2005).

The A10 nucleus has two modes of DA transmission based on the pattern on action potential firing: The *phasic* mode is driven by burst firing (up to 15–30 Hz), which results in rapid, considerable increase in DA concentration in the synaptic cleft in the terminal fields. However, under baseline conditions most of the released neurotransmitter is reclaimed by the dopamine transporter (DAT) (Grace et al., 2007). The *tonic* mode (2–10 Hz), is vital for normal functioning of neural circuits (Garris et al.,1997; Schultz et al., 1997; Schultz, 2007). It has much slower time course and thus facilitates the maintenance of the extracellular DA baseline level. The interactions of these two systems are considered to guide goal-directed behaviour and DA is traditionally regarded as a neurobiological substrate of reward and accordingly mesocortocolimbic system as a part of the reward circuit (Bratcher, et al., 2005; Schultz, 1997).

It should be noted that the concept of reward in the behavioural context is wider than the mere hedonic impact of stimuli. Instead, it is defined as any object or event that generates approach behaviour and consumption, induces learning of such behaviour and is a result of decision making (Berridge and Robinson, 1998; Schultz, 2007). Therefore, the role of DA in the reward circuitry includes a broad range of behavioural components such as learning, cognition and motivational salience, a process through which stimuli becomes the focus of goal-directed behaviour (Bromberg-Martin et al., 2010; Kapur, 2004; Steinberg and Janak, 2013). Recently, reports have proposed an additional role related to mediating heterogeneous responses to aversive stimuli, such as footshocks (Brischoux et al., 2009; Lammel et al., 2012).

Alterations in the synthesis, release or uptake of DA result in an abnormally

functioning reward system. Indeed, the dysregulated DA transmission has been linked to the pathophysiology of several neurodevelopmental, neurodegenerative and psychiatric diseases, as well as genetic disorders (Dichter et al., 2012; Prakash and Wurst, 2006). All of the disorders have been extensively discussed elsewhere, thus this review is limited to the major findings (Table 1). The neurodegenerative disorders vary from the other classes because they are associated with a reduced number of DA cells, while the other diseases are believed have a stereotypic hyperstimulated DA system of the left hemisphere, except depression, obsessive compulsive and attention deficit hyperactivity disorder, where DA transmission is in fact decreased. The susceptibility to these hyperdopaminergic disorders is considered to be dependent on development, thus the timing of prenatal insults affects the disorder type; insults during the first or second trimesters increase the risk for autism, whereas the second trimester or hypoxia at birth increase the risk for schizophrenia. Regardless of this difference, the disorders often have a high comorbidity (Dichter et al., 2012; Previc, 2007).

### 2.1.3. Classification of VTA DA neurons

VTA contains approximately 10500 DA neurons in mice, which are generally differentiated from other neuron types by using the expression of tyrosine hydroxylase or by the presence of hyperpolarization activated inward current ($I_h$), although the latter is considered controversial (Jomphe et al., 2005; Lammel, et al., 2011; Nelson et al., 1996). Despite the stereotypic anatomical locations, the properties of the DA neurons are not homogenous (Björklund and Dunnett, 2007). Indeed, the pharmacological, electrophysiological and molecular properties and corelease of other peptides, vary depending on which region the neurons project. Furthermore, the different subtypes are modified differently by rewarding and aversive stimuli. Therefore, the projection targets are often used to classify the neurons (Di Salvio et al., 2010; Lammel et al., 2008; Lammel et al., 2011; Ungless and Grace, 2012). Another categorisation has been establised according to cellular mophology and immunocytochemistry: Type 1 neurons are medium to small sized with proximal and distal varicosities whereas type 2 neurons are medium-sized and rarely show distal varicosities (Sarti et al., 2007).

**Table 1 Summary of the implications of DA neurons on human disorders.**

| Implications | Findings | References |
|---|---|---|
| Neurodegenerative disoders (Parkinson's disease (PD), Huntington's disease (HD)) | Degeneration of VTA neurons (more severe in substansia nigra) shown as cognitive dysfunctions such as symptoms of depression | Blonder & Slevin, 2011; Dauer and Przedborski, 2003; Hirsch, 1992 ; Wang et al., 2012 |
| Mood disoders (Major depressive disorder, Bipolar disorder) | Reduced mesolimbic and DA release activity during reward anticipation and reward learning. Increased in mania leading to heighted responses to positive emotions and excessive goal pursuit shown as abnormally increased energy levels and mood. Proposed genes regulated by DA: CREB, dynorphin, BDNF, MCH, or Clock | Coque et al., 2011; Cousins et al., 2009; Dailly et al., 2004; Drevets et al., 2008; Nestler and Carlezon, 2006 |
| Eating disorders (Bulimia, Anorexia nervosa) | Dopaminergic signalling increased by reduced leptin signalling shown as increased physical activity and feeding restriction. Altered allelic frequencies for DAT and DA receptor genes | Dichter et al., 2012; Fladung et al., 2013; Verhagen et al., 2011 |
| Schizophrenia | Imbalance with hyperstimulation of striatal neurons through dysregulated presynaptic control (positive symptoms of hallucinations) and hypoactive mesocortical projections to the PFC (negative symptoms of social withdrawal, and cognitive impairment). Relevant variants: DRD4, DRD2, PPP3CC, VMAT | Howes and Kapur, 2009; Kapur et al., 2005; Shi et al., 2008 |
| Attention deficit hyperactivity disorder (ADHD) | Altered sensitivity to positive reinforcement through downregulated tonic DA activity causes inattention and impulsivity. Relevant variants: DAT1, DRD4, DRD5 | Dichter et al., 2012; Tripp and Wickens, 2008; Volkow et al., 2009 |
| Substance abuse disoders (SUD) | Acute drug administration robustly increases DA transmission and chronic exposure reduces it in projections to limbic forebrain | Di Chiara and Imperato, 1988; Nestler, 2005; Volkow et al., 2009 |

| | | |
|---|---|---|
| Obsessive-compulsive (OCD), impulse control (ICD) disorder | Attenuated activation in projections to nucleus accumbens in reward anticipation causes decreased the ability to make beneficial decisions and repetitive behaviours (OCD). Attenuated activity to orbitofrontal cortex and anterior cingulated shown as compulsive gambling eg. Dopamine agonist treatment associated with ICD in Parkinson's patients | Figee et al., 2011; Voon et al., 2011; Weintraub et al., 2006 |
| Autism spectrum disorders (ASD) (Autistic disorder, Asperger's syndrome, Rett's syndrome, disintegrative disorder, pervasive developmental disorder (PDD), Fragile X syndrome (FXS)) | Overactivity of DA neurotranmission leading to severe social deficiets and hypervigilance. Elevated prenatal DA increases the risk for ASD. FXS condition results from unexpressed FMR1 which is suggested to regulate DA signaling cascades. Responses to rewarding stimuli not assessed to date | Dichter et al., 2012; Kohls et al., 2012; Previc, 2007 |
| Prader-Willi syndrome, Angelman syndrome | Overactivity of DA neurons through lack of GABAergic inhibition linked to excessive apetite arousal (Prader-Willi syndrome), Altered dopamine system regulator experiments indicate a link to the characteristic symptoms of prominent smiling and an attraction to water and certain types of paper and plastics. Empirical evidence is still lacking (Angelman) | Dichter et al., 2012; Dimitropoulos et al., 2000; Philpot et al., 2011 |
| Williams syndrome | Dysfunctional modulating of reward-responses to social cues shown as positive bias in recognition of facial expression and hypersociability | Doyle et al., 2004; Mimura et al., 2010 |

## 2.2. Glutamatergic modulation of VTA DA neurons

VTA receives regulatory input through interconnected network of afferents from various brain areas and by several systems including DA, GABA, acetylcholine, serotonin, endocannabinoids and orexin (Hikosaka et al., 2008). Here, the focus is on the glutamatergic modulation, which for the VTA is thought to originate from widely distributed brain regions. The most relevant for DA neurons are the prefrontal cortex (PFC), pedunculopontine tegmentum (PPTg) of the brainstem and lateral preoptic–rostral hypothalamic area (lPOA-rHA) as well as local VTA glutamatergic neurons (Geisler and Wise, 2008; Grace et al, 2007;

Omelchenko and Sesack, 2007).

### 2.2.1. Glutamate receptors

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system **(**CNS) acting through both ligand-gated ion channels and G-protein coupled receptors (Dingledine et al., 1999). This investigation focused on the ion channels, which have been pharmacologically divided into three classes based on their agonists: N-methyl-D-aspartate (NMDA), α-amino-3-hydroxyl-5-methyl-4-isoxazo-lepropionicacid (AMPA) and 2-carboxy-3-methyl-4-isopropenylpyrrolidine (kainate) receptors (Figure 2). The receptor groups are encoded by distinct gene families. However, based on the sequence similarity of receptor subunits and in some cases on the resemblance of intron positions, the ionotropic receptors have been suggested to have common evolutionary origin. In addition, all of the aforementioned are cation-selective allowing the passage of $Na^+$ and $K^+$, and in some cases $Ca^{2+}$ ions, which in turn increase the probability of action potential firing in the target neuron (Dingledine et al., 1999; Ozawa et al., 1998; Palmer et al., 2005).

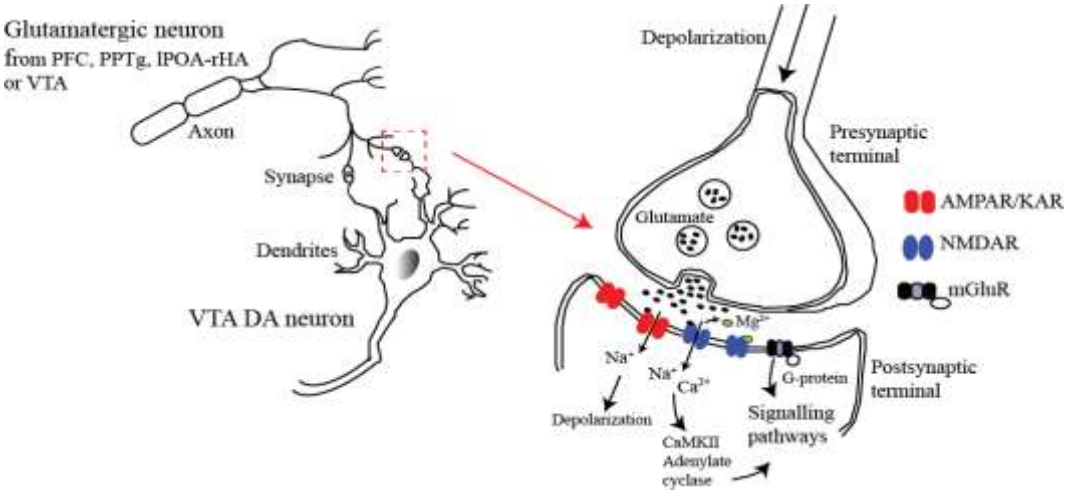


**Figure 2 Glutamatergic modulation of VTA DA neurons.** Activity of A10 nuclei is regulated by glutamatergic afferents originating from widely distributed brain regions and the VTA itself. Glutamate is released from its vesicles to the synaptic cleft upon depolarization of the presynaptic neuron. The neurotransmitter binds to its ligand-gated ion channels (AMPA/KAR and NMDAR) and G-protein coupled receptors (mGluR). Opening of the ion channels causes an influx of cations which subsequently activates downstream signaling pathways and thus modulates the activity of DA neurons. Abbreviations: PFC, (prefrontal cortex), PPTg (pedunculopontine tegmentum), lPOA-rHA (lateral preoptic–rostral hypothalamic area)

The vast majority of fast excitatory synaptic transmission is mediated by AMPA receptors (AMPAR) (Palmer et al., 2005). AMPARs, like other ionotropic

glutamate receptors, are tetrameric assemblies of subunits GluA1-4. The GluA2 subunit is critical for determining the properties of AMPARs, because a post-translational modification (so called RNA editing) introduces an arginine residue that confers the channel impermeable to $Ca^{2+}$. Consequently, the GluA2-containing AMPARs exhibit a linear current-voltage relationship that reverses at 0 mV. In the absence of GluA2, the receptors are $Ca^{2+}$ permeable and blocked by endogenous polyamines at positive potentials thus transforming the current-voltage curve to inwardly rectifying. The expression of GluA2 is also developmentally regulated and it is known to increase during the first postnatal week (Bellone and Lüscher, 2012).

NMDA receptor (NMDAR) subfamily is composed of seven subunits, which form heteromeric tertameric structures with the obligatory subunit GluN1 combined with either GluN2A-GluN2D or GluN3A-GluN3B. The composition has been shown to be developmentally regulated, and accumulating evidence suggests that this type of glutamate receptor is not a static component of the postsynaptic membrane. Instead its function and expression have been proposed to alter depending of the activity of the synapse (Bellone and Lüscher, 2012; Kew and Kemp, 2005). Accordingly, the receptors are thought to diffuse laterally between synaptic and extrasynaptic sites. NMDARs are activated by binding of both glycine and glutamate; however the channel is not opened without the release of the $Mg^{2+}$ block which occurs when the postsynaptic membrane is depolarized by the fast AMPA-induced current. This is followed by the influx of cations (Gladding & Raymond, 2011).

The third glutamate receptor subfamily represents the kainate receptors (KARs). KARs are composed of two related subunit families, GluK1-3 and GluK4-5 which form either homomeric or heteromeric structures (Dingledine et al., 1999). In conjunction with AMPARs, the receptors can flux $Ca^{2+}$ until an arginine residue is introduced. At postsynaptic sites, KARs mediate a minor element of excitatory postsynaptic currents while at presynaptic sites they exert a potent regulation on transmitter release at both excitatory and inhibitory synapses. The receptors are developmentally regulated and play pivotal roles in several processes including neuronal migration, differentiation and synapse formation (Cherubini et al., 2011;

Lauri and Taira, 2011). Owing to pharmacological similarities between KARs and AMPARs, the postsynaptic responses of these subfamilies are indistinguishable without specific selective antagonists (Chittajallu et al., 1999; Copits and Swanson, 2012; Coussen, 2009).

### 2.2.2. Proposed connections for synaptic plasticity regulation

Glutamatergic afferents are considered to be crucial for the functioning of VTA and an important regulator of its activity. Furthermore, their alterations have been reported to significantly alter DA release in the target regions which can consecutively lead to changes in the behaviour (Geisler and Wise, 2008; Mathon et al., 2003). In addition to inducing burst firing in DA neurons, glutamatergic afferents have been demonstrated to exhibit long-term potentiation (LTP) – a form of synaptic plasticity which renders the possibility to adapt to constantly changing environment through activity regulated synapse strengthening (Chen et al., 2010; Kauer and Malenka, 2007). In this mechanism, repeated activation of excitatory synapses evokes an increase in synaptic strength that can last for hours or even days. Generally, synaptic plasticity has been associated with learning and memory (Malenka and Nicoll, 1999).

In VTA the induction of LTP occurs after depolarization of the postsynaptic membrane and activation of NMDARs. The subsequent increase of intracellular $Ca^{2+}$ in turn activates several intracellular signaling cascades, most notably $Ca^{2+}$/calmodulin-dependent protein kinase II CaMKII related pathway (Kauer and Malenka, 2007). The strengthening is associated with increased trafficking of GluA2-lacking AMPARs into the postsynaptic spine, which in turn increases the sensitivity to glutamate (Chen et al., 2010; Kauer and Malenka, 2007; Lüscher and Malenka, 2011). It is noteworthy that, in the VTA GABAergic synapses are also capable of exhibiting LTP (Nugent and Kauer, 2008). Glutamatergic receptors are additionally important for the counterpart of LTP termed long term synaptic depression (LTD), which is associated with AMPAR withdrawal from the membrane. However, this form of synaptic plasticity does not require NMDA for the induction (Gutlerner et al., 2002; Hayashi et al., 2000; Kauer and Malenka, 2007).

## 2.3. Challenges of investigating the glutamatergic regulation

### 2.3.1. Modelling VTA *in vitro*

Work up-to-date suggests a crucial role for synaptic plasticity in early behavioural responses of drugs of abuse, as well as triggering long-term adaptation in the target regions (Kauer and Malenka, 2007). Owing to the central role DA has in the reward related behaviours and various disorders, the physiological properties of glutamatergic regulation and its alterations have been extensively studied using *ex vivo* and *in vivo* approaches (Geisler and Wise, 2008). However, using such methods makes it difficult to distinguish whether DA neurons are the primary target of the drug of interest. Thus, *in vitro* models could offer significant experimental advantages as such methods allow the presynaptic characteristics of DA neurons to be studied in isolation from systemic input from other brain regions thus simplifying the overlapping interactive circuits (Frank et al., 2008).

Literature describes several different approaches to model brain regions *in vitro* (Millet & Gillette, 2012). The highest resemblance to the 3D *in vivo* conditions is provided by organotypic cultures. The technique is based on growing tissue explants on culture media and it represents the intermediate method between acute brain slices and primary cell culture. While beneficial for long-term studies, organotypic cultures have disadvantages of higher experimental variation and they are considered less suitable for high-throughput screening (Benbrook, 2006; De Gendt et al., 2009).

Another *in vitro* model is dissociated primary culture which offers relatively unlimited access to individual mature neurons (Rayport et al., 1992; Fasano et al., 2008). The technique exploits enzymes and mechanical trituration to grow isolated neurons in appropriately conditioned culture dishes or coverslips, and the cultures can be established from mice or rats at different stages of development. Embryonic stage culture offers the advantage that mutation which are fatal at birth can be investigated to assess the subsequent development of the cells (Banker & Goslin, 1998; Kaech & Banker, 2006), This technique has been successfully used for midbrain DA neurons (Planken et al., 2010; Yu et al., 2008), however isolating only A10 nuclei is not reliable at this stage of the development (Kert Mätlik, personal

communications). Neurons derived from postnatal animals are considered to exhibit a relatively mature phenotype (Fasano et al., 2008) and was therefore the chosen tissue source for this thesis.

Several methodologies have been previously described for setting up a postnatal VTA culture (Congar et al., 2002; Fasano et al., 2008; Frank et al., 2008; Sulzer et al., 1998). A common approach is to culture the neurons together with glial cells to achieve a physiologically relevant environment and to increase the survival of neurons (Millet & Gillette, 2012). The simplest method to set up such co-culture is to plate the neurons on a previously established monolayer of astrocytes. The astrocytes are derived by isolating the forebrain, which is then enzymatically digested and mechanically triturated. The cells are grown in flasks to remove the remaining prefrontal neurons and microglia, which are known to secrete neurotoxic cytokines (Kaech & Banker, 2006). Upon reaching confluence, the enriched astrocytes are plated on culture wells or coverslips, which have been coated with polymers of basic amino acids to enhance the cell attachment (Congar et al., 2002; Jomphe *et al.*, 2005). As the division of glial cells is rapid, their proliferation is suppressed with a mitotic inhibitor when the astrocytes are confluent. The VTA DA neurons are then plated on top of the monolayer at appropriate concentration. The following day, the division of glial cells is again inhibited (Banker and Goslin, 1998; Fasano et al., 2008).

To visually identify the DA neurons, such cultures are often prepared from transgenic mouse line, in which the promoter of tyrosine hydroxylase is integrated with the gene for enhanced green fluorescent protein (EGFP) (Jomphe et al., 2005). The limitation of the culture system is that the proteolytic dissociation enzymes can alter the properties of some channels and receptors (Aikaike and Moorhouse, 2003). The alternative approach is to use vibration-based techniques to isolate the neurons from brain slices. The most popular technique is to use a micropipette, which is placed into a brain slice derived from a P1-P21 rodent. The tip is mounted on a piezoelectric component and vibrated parallel to the slice surface or lowered through the slice thickness (Jun et al., 2011; Vorobjev, 1991). When carried out correctly, single neurons still have functional presynaptic boutons attached. This method allows the rapid investigation of relatively mature neurons in a superiorly

controlled extracellular environment (Jun et al., 2011). Furthermore, due to the reduced dendritic trees present in such preparations, this technique facilitates more accurate measurements of current kinetics and voltage dependence of the studied neurons (Aikaike and Moorhouse, 2003).

### 2.3.2. The principle of whole-cell patch-clamp

The electrical events mediated by glutamategic receptors can be investigated using whole-cell patch-clamp technique, which was developed by Neher and Sakmann in 1976. Principle of the method is near-perfect electrical isolation of a small fraction of the cell membrane inside the tip of sharp glass pipette (Hamill et al., 1981). The experiments are performed in solutions that resemble the physiological ion contents of extracellular and intracellular milieus. Patch pipettes are mounted on a suction pipette holder and positioned on the cell surface using a micromanipulator. The tip is then pressed against the cell membrane and negative pressure is applied by suction to form a tight seal between the neuron and the pipette. Electric resistance of the junction exceeds 1 GΩ thus maintaining the level of background leak current caused by ion fluctuations between the cell and the pipette lower than 10 pA. This gigaseal facilitates the recording of crossing ionic currents even at the single channel level (Karmazínová and Lacinová, 2010; Möykkynen 2009).

The whole cell mode is achieved by applying additional short pulses of suction which ruptures the isolated patch, thereby establishing an electrical contact between the cytoplasm and a chlorided silver wire electrode placed inside the pipette which is filled with intracellular solution (Hamill et al., 1981). In this configuration, potentials are measured across the membrane with reference to ground electrode which is placed in the culture dish. During the experiments, neurons are constantly superperfused with extracellular solution, and receptors are activated by applying increasing concentrations of glutamate through local perfusion. The measurement of exact peak current requires conditions where the agonist application occurs extremely fast. Otherwise, the peak current results from a receptor pool containing some desensitised receptors (Möykkynen 2009; Karmazínová and Lacinová, 2010). The electrophysiology set up uses a feedback circuit to set the membrane potential to a desired command value. The opening of

glutamate-gated ion channels changes this command value, which is then automatically re-adjusted by the amplifier. This compensatory current is proportional to the current flowing through the ion channels and can be measured (Hamill et al., 1981; Möykkynen 2009; Walz et al., 2002).

# 3. Main aims of the thesis

The aim of this thesis was to investigate the distribution of DA neurons in 3-week-old and adult mice using Th-EGFP mouse strain and set up an *in vitro* model for VTA using primary culture and acute isolations. The third goal was to gain experience of electrophysiology by investigating the glutamatergic regulation of DA neurons using the whole-cell patch-clamp method.

# 4. Materials and Methods

## 4.1. Mouse strains

All experiments were performed using either transgenic Th-EGFP line (Gong et al., 2003) carrying the EGFP gene under the control of the Th promoter or wild type strain C57BL/6J (Charles River Laboratories, Germany). The mice were group-housed under a 12-h light/dark cycle with food and water available *ad libitum*. The Th-EGFP line was maintained as heterozygotes by crossing them to C57BL/6J. Therefore, offspring that carried the transgene were identified using tail sample PCR, using the primers: CCT ACG GCG TGC AGT GTC TCA (forward) and CGG CGA GCT GCA CGC TGC GTC (reverse). Both genders were used throughout the project. All experiments were carried out following animal protocols approved by University of Helsinki (Licence No. ESAVI0010026/041003/2010).

## 4.2. Expression map of Th-EGFP

### 4.2.1. Perfusion and fixation of brain samples

To perfuse the brains, animals were anesthetised using pentobarbital sodium (Mebunat, Orion Finland) (100-200mg/kg). Once an animal had reached the deep surgical state of anaesthesia, perfusion surgery was performed: Skin was cut around the thoracic cavity and muscles along with the rib cage were broken. Next, a small hole was made to the right atrium and the perfusion cannula was inserted in the left ventricle. After this, 50 ml of +4 °C phosphate buffered saline (PBS) containing (in M): 1.3 NaCl (Fisher BioReagents Cat. No. BP3581), 0.07 $Na_2HPO_4$ (Sigma-Aldrich, Finland Cat. No. S7907), 0.03 $NaH2PO_4$ x 2 $H_2O$ (Sigma-Aldrich, Finland Cat. No. S0751) was applied through the circulatory system to remove the blood (Univentor 864 Syringe pump). Tissue was then fixed by applying 45 ml of +4 °C 4% paraformaldehyde (PFA) (Sigma-Aldrich, Finland Cat. No. 158127). The animal was then decapitated and brain removed to 4% PFA for an overnight incubation. At this step, a maximum of six brains were used for 50 ml of PFA solution. Brains were then cryoprotected with 30% sucrose (Sigma-Aldrich, Finland Cat. No. S0389) dissolved in PBS solution, in which they were left to incubate until the floating was deceased. Samples were then frozen and

stored at -80 °C. In total 9 Th-EGFP transgenic mice from two age groups were used for this experiment: Adult (10-week-old) (n=5) and P21 (3-week-old) (n=4). The retinal A17 population was not included in the analysis.

### 4.2.2. Slice preparation and immunohistochemistry

The brain samples were cut to 40-µm-thick slices using a cryostat (Leica) set to -20 °C. This was done by taking two replicas for every third slice and then storing on the polylysine-coated slides (Fisher Scientific Cat. No. J2800AMN2) in -20 °C. Only those areas containing dopaminergic cell populations A8-A16 and the subpopulations vlPAG and DNR were included in the experiment and used for immunostaining. This was carried out by equilibrating the thawed slices in PBS after which they were stained for 10 min with 0.02mg/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Finland Cat. No. D9542) dissolved in PBS solution. Slides were then washed three times with PBS for 5 min, mounted with Vectashield (Vector Laboratories, Cat. No. H-1000) and stored in dark at RT.

### 4.2.3 Imaging and statistical analysis

Photomicrographs of DA cell populations were captured using a light microscope with a 10x objective (Leica DMR, Germany) and Apotome camera. Only the samples with successfully preserved Th-EGFP signal were included in analysis. The number of GFP-positive neurons on one hemisphere was counted with ImageJ software for each animal using sampling regions of specific stereotaxic Bregma coordinates (in mm): A16 (4.28; 3.56); A14, A15 (-0.10; -0.22); A13 (-0.82; -0.94), A12, A11 (-1.82;-2.06), A9 (-2.92; -3.28), A10 (-3,40; -3.4), A8 (-4.04; -4,16); vlPAG (-4,72; -4.96) (Paxinos and Franklin, 2010). The number of positive cells was averaged for each sampling region and the statistical difference between the two age groups was assessed by Mann-Whitney U-test for each DA cell group.

## 4.3. Primary cell culture

In the literature, several different culture systems have been designed to grow VTA neurons *in vitro.* Here, several pre-existing unspecific methods were

modified and combined to set up an optimal system for the available cell culture facility which would also serve to overcome its marginal technical limitations (Figure 3). This part of the project was done in collaboration with Professor Dan Lindholm's group. Primary cultures were maintained in a humidified atmosphere of 5% $CO_2$ at 37 °C and all solutions and equipment were sterilised either by autoclaving or filtering.

### 4.3.1. Adhesion substrate preparation

Prior to plating the astrocytes, 22-mm coverslips (Fisher Scientific Cat. No. 11390675) were washed with 95% ethanol and then twice with 70% ethanol. As alcohol burner was not available in the cell culture facility, the coverslips were either left to dry or rinsed with D-PBS (Sigma-Aldrich, Finland Cat No. D5652). The coverslips were then coated with polymers of basic amino acids. Here, three different coating procedures were tested based on the literature and reagents present in the facility: Poly-D-lysine (Sigma-Aldrich, Finland Cat No. P7280) (Banker & Goslin, 1998) was diluted with $ddH_2O$ to a 5 µg/ml working solution, which was incubated for 2 h at RT after which coverslips were washed three times with $ddH_2O$. Poly-L-lysine (Sigma-Aldrich, Finland Cat No. P4707) was diluted to 100 µg/ml with both $ddH_2O$ (Banker and Goslin, 1998) and borate buffer (Appendix 2) and incubated for 1 h. After the incubation the coverslips were washed by plunging them sequentially into 3 beakers of $ddH_2O$ after which they were placed on Whatman paper and 65 µl of $ddH_2O$ was placed on each coverslip. The slices were incubated for 1 h, the procedure was repeated, and the slices were incubated for another 1h. Then, excess $ddH_2O$ was aspirated off and the slices were allowed to dry completely (Fasano et al., 2008). The third tested procedure was to incubate the coverslips for 3 h at 37 °C in 5mg/ml poly-L-ornithine (Sigma-Aldrich, Finland Cat No. P4957) D-PBS solution and rinse the coverslips three times with $ddH_2O$ (Celine Bruelle, personal communication).

All the polymer solutions were used with volumes sufficient to cover the entire surface of the coverslips, which was approximately 100 µl. Identical coating procedures were used when cell were plated directly onto the 35x10mm culture dishes (Thermo Scientific Cat. No. 130180), however the poly-L-lysine working

dilution was 50 µg/ml (Neuvitro instructions) and wells were not sterilized prior to coating.

### 4.3.2. Astrocyte monolayer preparation and maintenance

For the astrocyte primary culture, a P0-P3 mouse pup from any strain was decapitated and the cortex was removed according to a protocol published by the Sulzer lab (Sulzer and Kanter, 2011) with the modifications that Sylgard circles were not used and the procedure was done in dissociation solution instead of PBS. Brain segments were then digested either with heat-activated papain (Sigma-Aldrich, Finland Cat. No. P4762) solution (Appendix 2) or with trypsin (Sigma-Aldrich, Finland Cat. No. T4424) which was diluted to 2.5% in D-PBS. Brain segments were allowed to incubate either for 50 min with submersible agitation or 20 min without the agitation. The segments were then washed, triturated and plated to culture bottles according to Fasano et al., (2008) except that inhibitory solution was not used and glass pipettes were replaced with plastic pipette tips (Biohit) and the 25-$cm^2$ and 175 $cm^2$ culture flasks were replaced with 75-$cm^2$ culture flasks (Thermo Scientific Cat. No.130190). Thus, the cell suspension was divided to 15 ml to form monolayers and 5 ml to produce the conditioned media.

The cultures meant for conditioned media were allowed to grow for 14 days after which their media was harvested and stored at 37 °C. This was later used as a neuronal medium supplement. To remove unwanted microglia and prefrontal cortex neurons, the astrocyte cultures were washed the next day with cold medium according to Fasano et al., (2008) with the adjusted volumes: Washing was carried out twice with 3 ml of cold medium which was then replaced with either 8 ml (monolayers) or 40 ml (conditioned media).

A subset of the cultures was purified with a different protocol by allowing the cells to grow into a confluent monolayer, after which the cultures were agitated on an orbital shaker (Heidolph 1000) and then replated (Schildge, et al., 2013). This was carried out by first agitating the cells at 180 rpm for 3 min. The media was then discarded and replaced by 20 ml. The next agitation step was 6h at 240 rpm. After

this, cells were vigorously agitated by hand for 1 min and rinsed twice with PBS to remove traces of serum. Then, D-PBS was replaced with 2 ml of trypsin that was incubated for 2-5 min. Next, 5 ml of astrocyte medium was added to quench the trypsin, and the solution was triturated 10 times. Cells were then pelleted by centrifugation (1000g, RT, 5 min), resuspended in 10 ml of astrocyte medium and counted using a hematocytometer to be plated at 100000 cells/ml. The medium was replaced every 2-3 days in both procedures and two different media-supplement combinations were tested for the culture: Basal Medium Eagle (Sigma-Aldrich, Finland Cat No B1522) (Jomphe et al., 2004) and Minimum Essential Medium (Fasano et al., 2008) (Appendix 2).

The astrocytes were transferred from flasks to wells or coverslips after they had reached confluence (approximately 7 days). This was again carried out with trypsin and identical plating density. After the enriched astrocytes had grown into confluence, their division was inhibited by adding 12.5 µl 5-Fluoro-2-deoxyuridine (FUDR) solution per well (Appendix 2). The monolayers could then be kept for 2-3 days before use. One astrocyte culture could be used for three weeks. Therefore, the alternative option was to re-plate the cells in a fresh 75 $cm^2$ culture flask and let them grow until confluence.

### 4.3.3 VTA dissociation and co-culture maintenance

One day before plating the VTA neurons onto the astrocyte monolayes, the astrocyte medium was replaced with appropriately supplemented Neurobasal-A medium to condition the monolayer for neurons. As the Frank et al., (2008) neuronal media was significantly different from Fasano et al., (2008) and would have thus required several additional - perhaps unnecessary - chemicals to be ordered, all of the experiments were carried out with Neurobasal-A supplemented with penicillin/steptomycin (Life Technologies, Gibco Cat No 15070-063), GlutaMAX to minimize the toxic effects of ammonia build-up (Life Technologies, Gibco Cat No 35050-038), B27 to promote growth (Life Technologies, Gibco Cat No. 17504044) and Fetal bovine serum (Life Technologies, Gibco Cat No. 26140-079)

The mesencephalic DA cultures were prepared by harvesting the brains from P0-P2 animals. The initial experiments were carried out with C56BL/6J mice and the established protocol was then tested with Th-EGFP animals. The dissection procedure has been extensively described in the literature (Fasano et al., 2008; Frank et al., 2008). In summary, a 1-2 mm coronal slice was cut at the level of the midbrain flexure and segments dorsal, lateral and ventral to the VTA were removed. Three brains were harvested at a time and kept in ice-cold dissociation solution (Appendix 2) throughout this procedure. As carbogen source was not available at the cell culture facility, the papain incubation was only carried out according to Fasano et al., (2008) instructions. Thus, the brain segments were incubated for 20 min both with and without submersible agitation. The trituration and plating procedures on the other hand were tested with three different treatment combinations. Depending on size of the animal, one brain was sufficient for 1-2 monolayers of astrocytes when the plating was done at 240000 cells/ml.

**Protocol 1. Purification by centrifugation**

The first option was to follow the complete Fasano et al., 2008 protocol, which includes a centrifugation step and the use of solutions with different compositions of Neurobasal-A, a trypsin inhibitor (Sigma-Aldrich, Finland Cat. No. T6522), bovine serum albumin (BSA) (Sigma-Aldrich, Finland Cat. No. 04503) and HEPES (Fisher BioReagents Cat. No. BP310). However, the sedimentation step was carried out with significantly lower speeds to minimize mechanical stress. As such settings were not possible with the available equipment, this step was executed by centrifugation (400g, RT, 2 min) and (900g, RT, 3min). The neurons were then resuspended and plated.

In all of the three protocols, 12 µl of FUDR was added to the co-cultures after 24h incubation. The cultures were then left to incubate with as little disturbing as possible for the next 7 days after which they received 500 µl of supplemented Neurobasal-A and 10 µl of kynurenic acid (Sigma-Aldrich, Finland Cat. No. K3375). From this point forward the cultures received fresh 500 µl of Neurobasal once a week which was was supplemented further by combining it with the harvested astrocyte conditioned medium (33ml of conditioned media to 66 ml Neurobasal-A).

**Protocol 2. Purification by media changing and subsequent incubation steps**

The second protocol option was to follow the trituration of plating instructions of Frank et al., (2008) with the modification that slide rings were not used for the plating. Here, the segments were washed three times with warm medium that was discarded each time after 3 min incubation. Neurons were then triturated with three different tip sizes by pipetting 25 times. The resulting solution was collected each time and plated with the same density that was used in protocol 1. These cultures received 100 µl of 2.08µg/ml glial derived neurotropic factor (GDNF) (Life Technologies, Cat. No. PHC7044) solution immediately after plating, and were left to incubate for 7 days. Media changing regime was done according to protocol 1, however these cultures did not receive conditioned medium or kynurenic acid.

**Protocol 3. Purification by a nylon strainer**

Considering that the second protocol did not include a step to remove cellular debris, the third tested option was otherwise similar except that the collected cell suspension was poured through a 40-µm nylon strainer (BD Biosciences Cat. No. B9320) prior to plating (Celine Bruelle, personal communication). Co-cultures prepared with this method were tested with both conditioned media supplemented and non-supplemented Neurobasal-A, with the changing interval of one week.

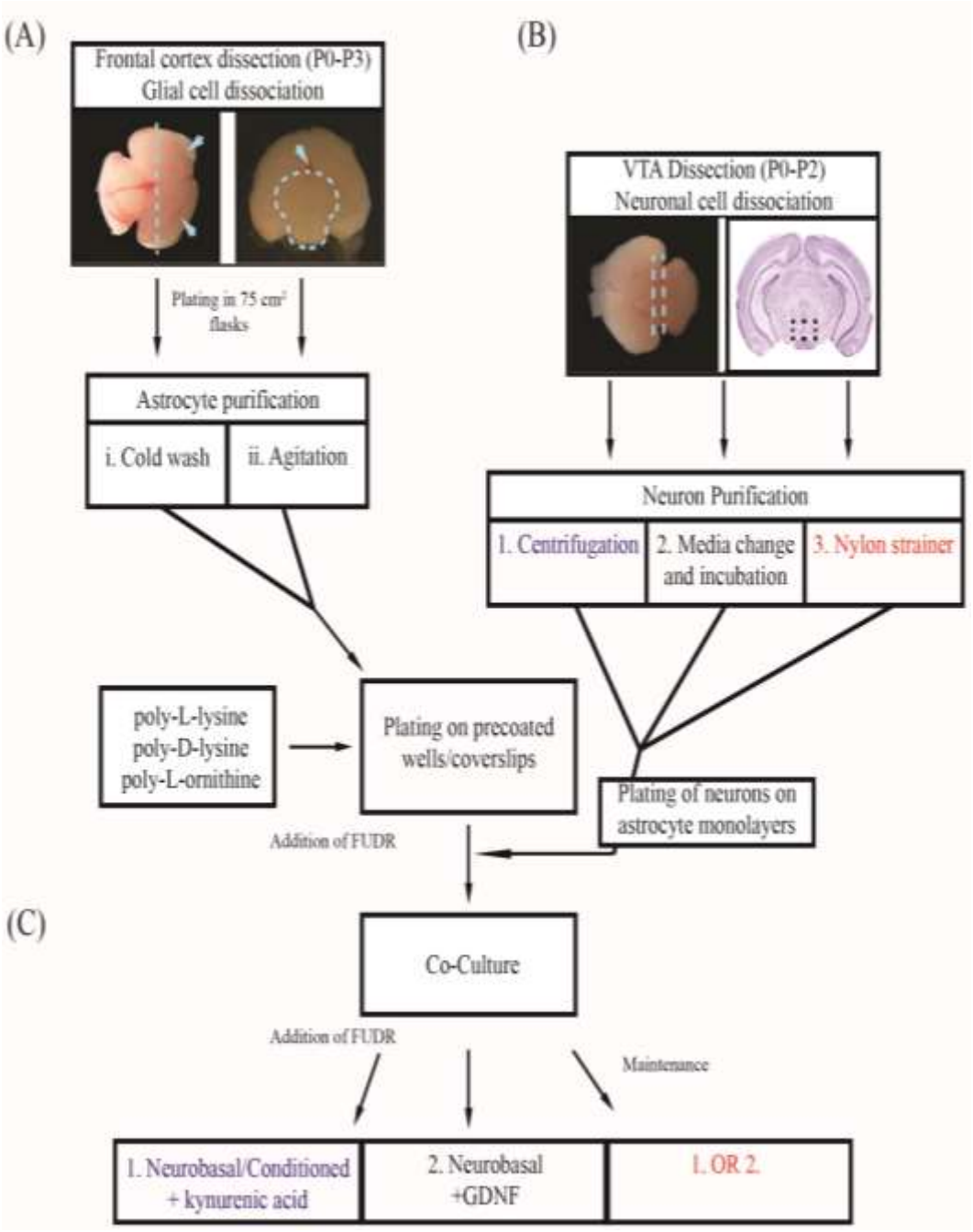


**Figure 3 Flow chart of the primary cell culture protocol.** Astrocytes were dissected from the frontal cortex following the dashed lines. Meninges, indicated by the blue arrows, were carefully removed and papain dissociated cells were plated on culture bottles. The astrocyte purification was carried out either through cold was (i) or agitation on an orbital shaker (ii) and cells were plated onto coated wells or coverslips. Once they reached full confluence, the division was halted (A). VTA neurons were dissected following the dashed lines and the papain dissociated neurons were purified by three different protocols. Antineoplastic agent was again added after they were plated on the astrocyte monolayers (B). The co-cultures were maintained by three different protocols depending on how the neurons were purified (C). Images adopted and modified from Fasano et al., (2008) and Paxinos and Franklin (2001)

## 4.4. Immunofluorescence of cells

### 4.4.1. Antibodies

The following antibodies were used in this study: anti-TH rabbit polyclonal antibody (Millipore, Cat. No. AB152) visualised with anti-rabbit Alexa-568-labeled antibody (goat) (Molecular Probes, Cat No A-11011), anti-tubulin β3 mouse polyclonal (Millipore Cat. No. 92590) visualised with anti-mouse Alexa-488-labeled secondary antibody (goat) (Molecular Probes, Cat No. A-11001). All of the antibodies were diluted at 1/1000 in 2.5% BSA.

### 4.4.2. Fixation and staining of neurons

Cultured neurons grown on glass coverslips were fixed with 4 % paraformaldehyde in $ddH_2O$ for 20 min, rinsed twice with D-PBS and permeabilised for 5 min with 0,25% Triton X-100 (Thermo Scientific Cat. No. 85111) in D-PBS (Banker & Goslin, 1998). The coverslips were blocked with 10% BSA for 10 min at RT and the diluted primary antibodies were incubated for 1h at RT or overnight at +4 °C. The secondary antibodies were incubated for one hour at RT. The coverslips were washed three times with D-PBS after all antibody incubations and then mounted using Vectashield. Images were obtained using a light microscope with a 20x or 40 x objectives (Leica DMR, Germany) and a CCD camera (Leica DC 300). A subset of cultures was stained with and inverted method, where the solutions were added directly on the bottom of the well and the coverslips were eventually placed on top of the preparation and imaged.

## 4.5. Mechanical isolation

Mechanical dissociation procedure has been previously described in the literature (Jun et al., 2011; Ye et al., 2004; Vorobjev, 1991). In this study, several protocols were combined and modified to acutely obtain VTA neurons with intact functional synaptic terminals (Table 2).

### 4.5.1. Brain slice preparation

Horizontal midbrain slices (250-300 μm) were obtained from P0-P7 or P14 C57BL6 mice using a vibrating blade microtome (Vibratome 1000 plus). During

this procedure, the brains were immersed with ice-cold and 95% O2 and 5% CO2 oxygenated cutting solution, which was either sucrose or artificial cerebrospinal fluid (ACSF) solution containing (mM): 124 NaCl, 4.5 KCl (Amresco Cat. No. 0395), 1.2 $NaH_2PO_4$, **(**Amresco Cat. No. 0571), 10 D-glucose (Amresco Cat. No. 0188), 26 $NaHCO_3$ (Amresco Cat. No. 0865) 1 $MgCl_2$ (Sigma-Aldrich, Finland Cat. No, 31431), 2 $CaCl_2$ (Amresco Cat. No. 0556) (Jun et al., 2011). After the cutting, the slices were allowed to recover for 1 h on a grid placed in an ACSF filled beaker, which was kept either at 37°C or RT (Figure 4). After this, the samples were transferred one at a time to a standard 35 mm culture or to a dish coated with poly-L-lysine or poly-L-ornithine. The slice was held down using a bent platinum wire and the VTA was identified under a binocular microscope (Lomo, MBC-10).

### 4.5.2. Vibrodissociation

Acute isolation was carried out using custom-built equipment (Figure 4 and Appendix 1). To achieve this, 1.5 mm diameter borosilicate glass capillaries (World Precision Instruments, Stevenage, UK) were formed into an L-shape with a Bunsen burner, pulled with a horizontal micropipette puller (P-87 Sutter Instrument Co., Novato, CA) to create a sharp tip, or pulled with a subsequent fire polishing step (Narishige Micro Forge 830, Japan) to create a fused ball-shaped tip with a diameter of 200-300 μm. The micropipette was then mounted onto a piezoelectric component attached to a pulse generator. This was tested with two options: A ceramic bimorph attached to a custom-built generator (Courtesy of Juha Voipio, University of Helsinki), and a relay attached to a Kenwood FGE 1202 sweep function generator (Courtesy of Organic and Nanoelectronics group, Tampere University of Technology).

The micropipette was guided above or on the surface of the slice with the attached manipulator. With the custom-built generator, the frequency was chosen at the level which created the largest lateral travel distance whereas the Kenwood generator was used at 20-50 Hz frequencies. The duration of the vibration treatment was also varied from 10s to 1 min, during which the micropipette was either kept above the slice, on its surface or it was moved deeper into the tissue such that it passed through the entire slice. After the treatment, the slice was shaken and removed with

forceps and the neurons were allowed to adhere to the bottom of the dish for at least 10 min prior to further experiments.

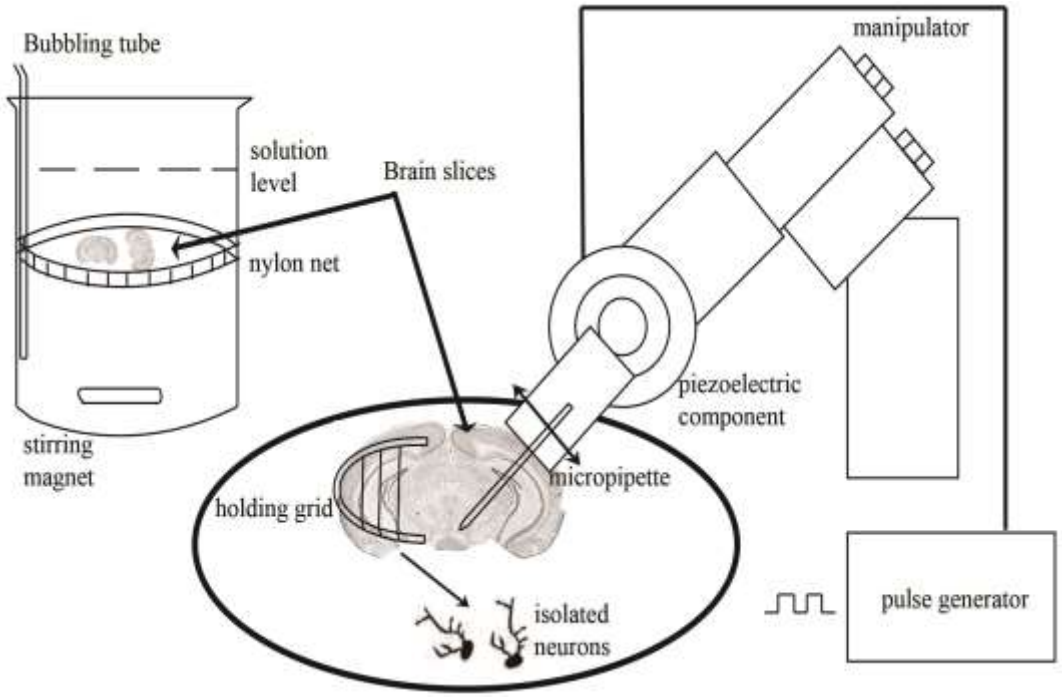


**Figure 4 Schematic diagram of the slice incubation chamber and vibrodissociation set up**. Obtained midbrain slices were left to recover for 1h in solution saturated with 95% $O_2$ and 5% $CO_2$. Slices were then transferred to a culture dish and held down with a grid. Manipulator connected micropipette was positioned onto VTA and vibrated laterally by a pulse generator connected piezoelectric component at various frequencies and durations. Brain slices was removed after the treatment and the dissociated neurons were allowed to settle for a minimum of 10 min prior to further experiments (Not drawn to scale) Brain slice figure adopted from Paxinos and Franklin (2001).

**Table 2 Summary of the optimised parameters for mechanical dissociation**.

| Brain slice preparation | | | | |
|---|---|---|---|---|
| Parameter | Age | Cutting solution | Temperature | Coating |
| Tested | P0-P7 | Sucroce | 37 °C | poly-L-lysine, poly-L-ornithine |
| | P21 | ACSF | RT | nothing |
| **Vibrodissociation** | | | | |
| Piezoelectricity | Pulse genetor | Time/Frequency | Tip shape | Tip position |
| Bimorph | Custom-made | 10s-1min | L (unpulled) | Above, on the surface |
| Relay | Kenwood | 10-50 Hz | Sharp, fire-polished | passing through |

## 4.6. Whole-cell patch-clamp electrophysiology

Electrophysiological recordings were performed at RT on EGFP-expressing

and non-EGFP expressing C57BL6 derived neurons maintained for 10-15 days in culture. The dishes were carefully washed several times to remove any traces of media prior to transfer to the recording stage of an inverted Olympus Provis IX70 epifluorescence microscope. Synaptic responses were measured using the whole-cell patch-clamp technique (Sakmann and Neher, 1984) (Figure 4 and Appendix 1). The mechanically isolated neurons were investigated with the same set-up.

### 4.6.1. Solutions

The extracellular solution consisted of (in mM): 140 NaCl, 5 KCl, 2 $MgCl_2$, 2 $CaCl_2$, 10 HEPES and 10 D-glucose. The pH was adjusted to 7.35 with 1 M NaOH (Metrohm 774) and the osmolarity was checked with an osmometer (KNAUER, semi-micro osmometer K-7400) and adjusted to 305 mOsm by adding sucrose. The ionic composition of the intracellular pipette solution was (in mM): 130 cesium methanesulfonate (Sigma-Aldrich, Finland Cat. No. C1426), 10 HEPES, 0.5 EGTA (Tocris Bioscience Cat. No. 2807), 8 NaCl, 5 QX314 (Sigma-Aldrich Cat. No. L5783**)**, 4 MgATP (Sigma-Aldrich, Finland Cat. No A9187), 0.3 MgGTP (Sigma-Aldrich, Finland Cat. No. G8877), and 10 BAPTA (Tocris Bioscience Cat. No. 2786) (Heikkinen et al., 2009). For this solution, the pH was adjusted to7.2–7.25 and osmolarity to 280 mOsm. The intracellular was frozen as aliquots, filtered prior to use and kept on ice throughout the recordings.

### 4.6.2. Recording

The cells were constantly superfused with the extracellular solution using a gravity flow system (Figure 5 and Appendix 1). Pulled borosilicate glass pipettes had a resistance of 2-5 MΩ when filled with the intracellular solution. Whole-cell patch-clamp recordings were made from VTA DA neurons at holding potential -70 mV with an EPC 9/2 double patch-clamp amplifier and pulse v 8.80 software (HEKA electronic, Lambrecht, Germany). L-Glutamic acid (Sigma-Aldrich, Finland Cat. No. G-2128) was dissolved in the extracellular solution at 4 different concentrations (in mM): 0.01, 0.1, 1, 10. After dissolving, pH was readjusted to 7.35. The agonist was applied through local superfusion from fused glass capillaries involving lateral movement of the tubing achieved by a stepper motor

driven solution application system (Warner Instrument, Hamden, CT). The agonist tube valves were opened manually with 5s intervals between different concentrations, and the wash tube remained open during all applications. Each application was done four times (except with 0.1mM in experiment 2 which was done 10 times) and the traces were averaged. The recordings were digitized using a Digidata 1322A analog to digital converter (Molecular Devices) with a sampling rate of 10 kHz and filtering using a lowpass bassels filter at 1kHz (Coleman et al., 2009). Only the neurons with full responses were included in the analysis that was carried out using Clampfit 10.2 (Molecular Devices, Sunnyvale, California) and Prism 3.0 software (GraphPad, San Diego, California).

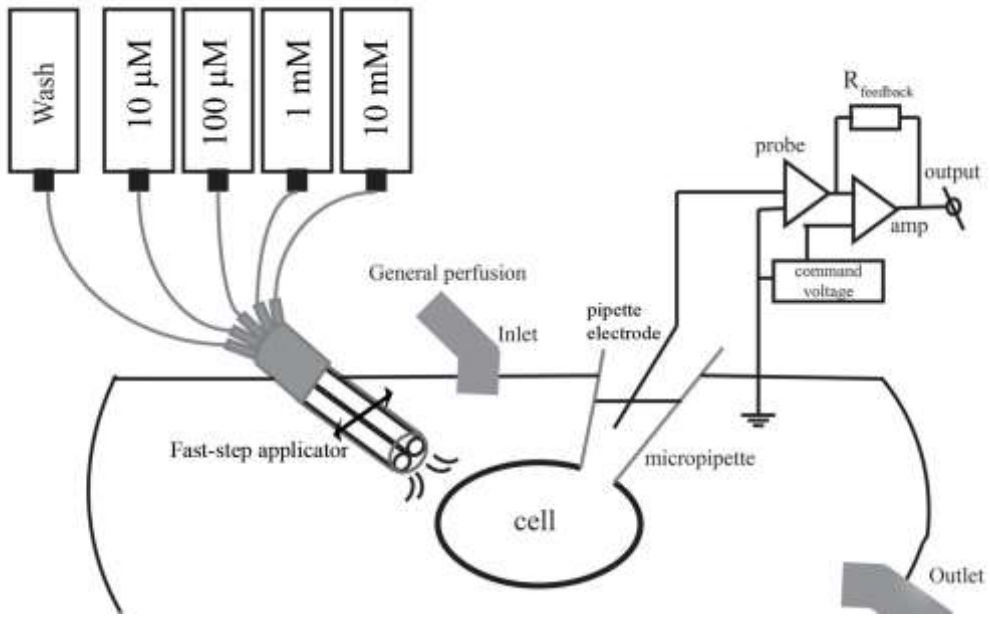


**Figure 5 Schematic diagram of the whole cell patch clamp set up.** The patched neuron was constantly submerged in fresh extracellular solution through the general perfusion system. Antagonist of increasing concentrations was directly applied to the cell through lateral movement of the fused glass tubes created by the fast-step applicator system. The wash tube remained open through the application. The changes of the membrane potential caused by the response to the agonist were measured against the command voltage and the resulting compensation current was recorded.

# 5. Results

## 5.1. Young and adult Th-EGFP mice exhibit a comparable A8-A16 phenotype

To evaluate the pattern of Th-EGFP expression, sections of perfused brains of adult and 3-week-old mice were counterstained with DAPI, imaged and the number of Th-positive neurons was counted from representative sampling regions from one hemisphere for each nuclei and subnuclei (2-3 sections per area). Out the 9 animals used in this experiment, 3 were included in the analysis from both age groups (Figure 7 and 8). The results show that all recognised A16-A8 nuclei, along with vlPAG and DRN subnuclei, can be identified in the Th-EGFP strain samples, and the different age groups show comparable phenotypes (Figure 6). In addition, there was no significant difference between the two age groups (Mann-Whitney U test p-value >0.127). In this study, the A15 group was only identified in the preoptic nucleus.

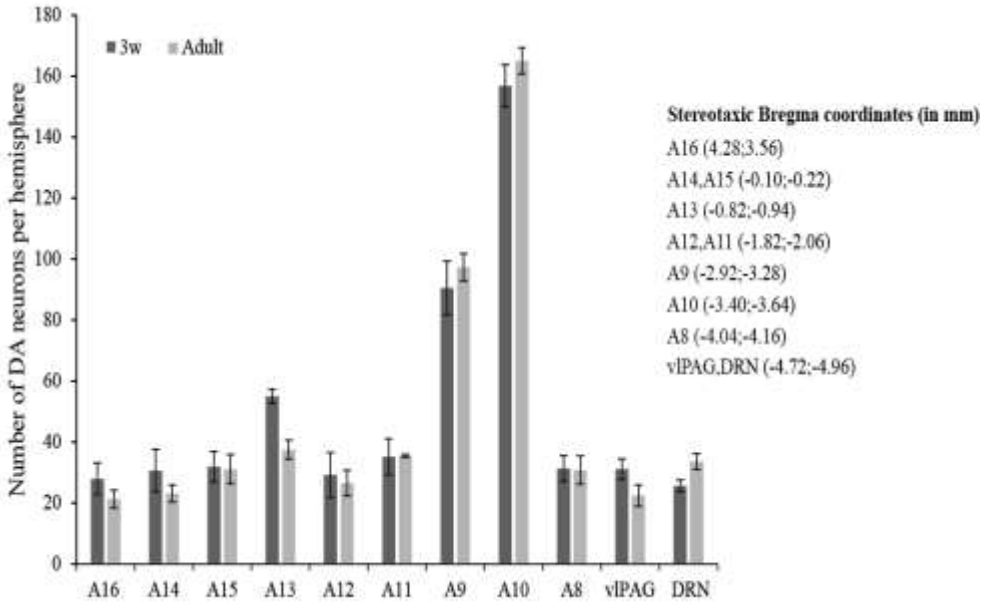


**Figure 6 Comparison of the number of Th-positive neurons present in one hemisphere in the sampling of A16-A8 nuclei, and subnuclei vlPAg and DRN in young and adult animals.** Error bars represent SEM-values.

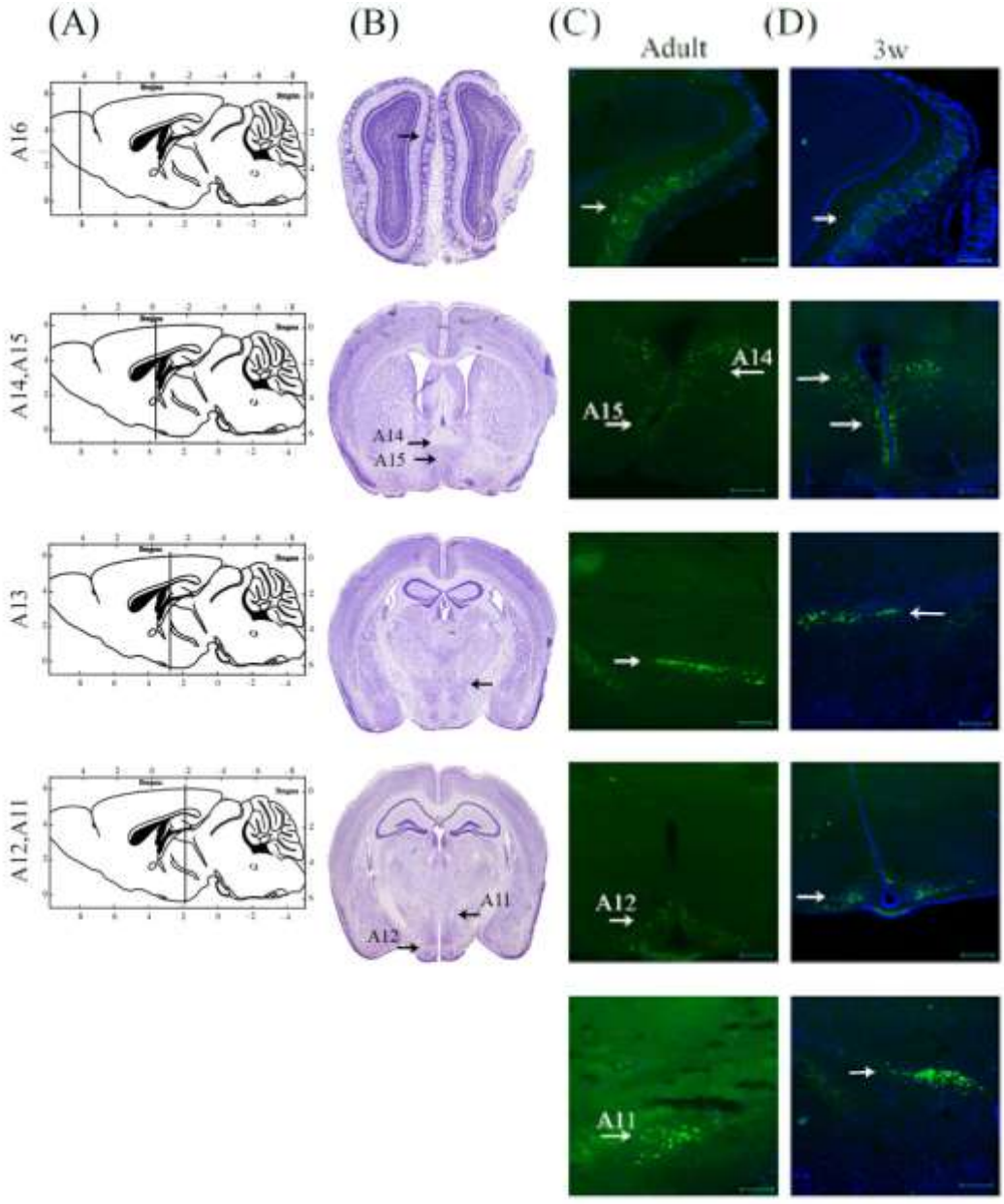


**Figure 7 Tyrosine hydroxylase expression in olfactory bulbs (A16) and diencephalon (A14-A11).** The level at which the coronal slices were taken are indicated by a vertical line on sagittal images (A) and nuclei localisation is indicated by arrows on coronal (B) images modified from Paxinos and Franklin (2001). Th expression in perfused in DAPI counterstained brain slices identifies the dopamine producing neuron populations A16-A11 in adult (C) and 3 week old (D) Th-EGFP mice. White arrows indicate the localisation of each population. Phenotypes were comparable in both age groups. Scale bar 200µm.

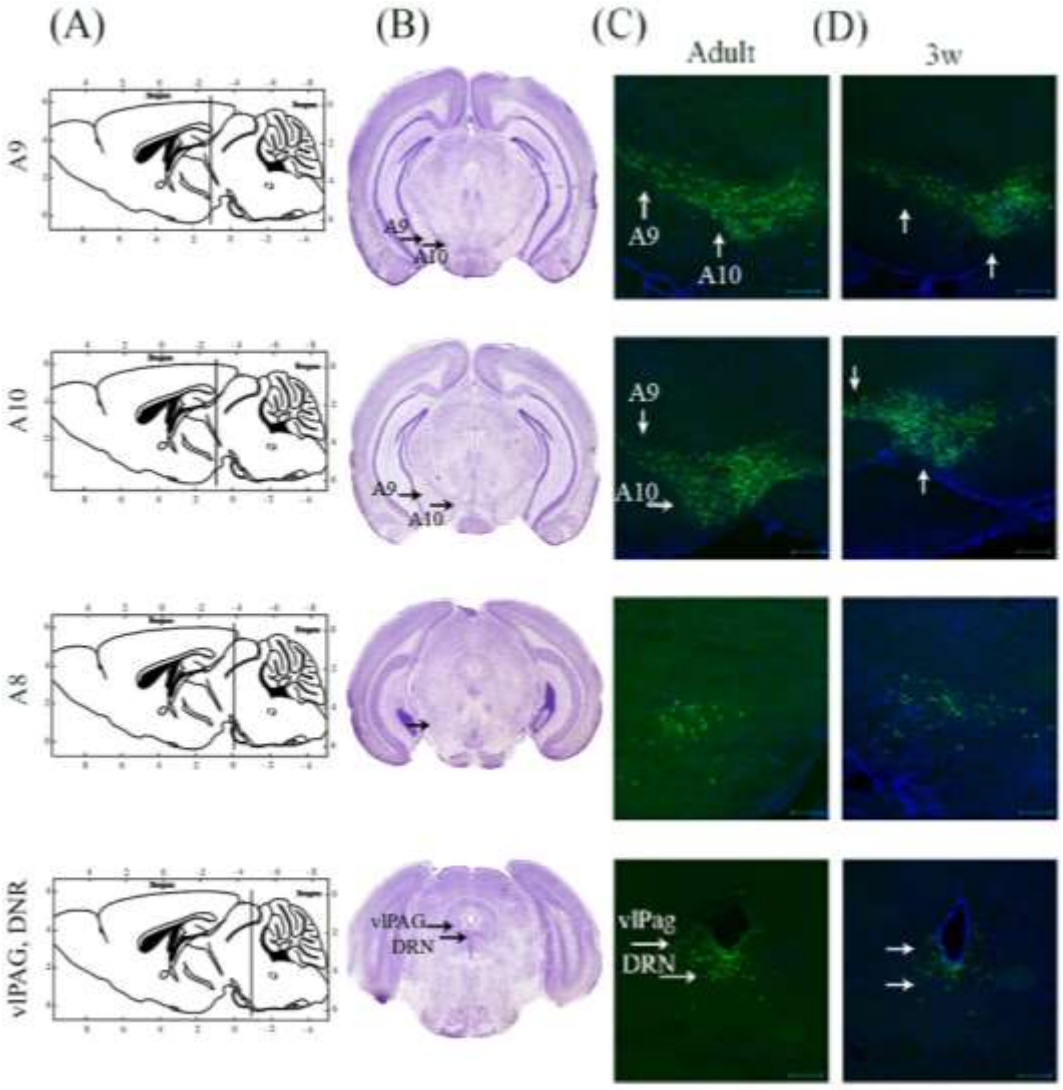


**Figure 8 Tyrosine hydroxylase expression in mesencephalon (A10-A8) and vlPAG, DNR subnuclei.** The level at which the coronal slices were taken are indicated by a vertical line on sagittal images (A) and nuclei localisation is indicated by black arrows on coronal (B) images modified from Paxinos and Franklin (2001). Th expression in perfused in DAPI counterstained brain slices identifies the dopamine producing neuron populations A910A8 and the subnuclei vlPAG and DNR in adult (C) and 3-week-old (D) Th-EGFP mice. White arrows indicate the localisation of each population. Phenotypes were comparable in both age groups. Scale bar 200µm.

## 5.2. Comparison of primary culture and acute dissociations as models for VTA

To study the physiology of DA neurons in a controlled environment, VTA was modelled with both acute dissociations and primary culture. The primary culture was obtained by establishing a monolayer of astrocytes by culturing cortical cells in flasks to remove unwanted glial cells and neurons. In this study, papain

digestation introduced a higher yield of astrocytes. Out of the two tested enrichment methods, the protocol involving an orbital shaker gave a marginally lower level of microglia yet such minimal improvement did not compensate for the required increase in preparation time. As the cold was enough to create a sufficient environment for the neurons, the rest of the experiments were carried out with the simplest purification method (Figure 9A). According to the results, the two tested media-supplement combinations are equally suited to support the growth of astrocyte monolayers.

The VTA DA neurons were obtained with all three described protocols; however the cells were most viable with either protocol 2 or 3 (Figure 9C and 9D). According to the results, the use of submersible agitation had minimal effect in all the protocols. The nylon strainer created a lower level of debris, however the difference was minimal. Out of the two media tested for this protocol, it was shown that supplementing Neurobasal-A with astrocyte conditioned media did not significantly improve the viability of the neurons. Instead it increased the necessary preparation time, and the risk for contaminating the culture. Furthermore, even without this additional conditioning, neurons prepared according to protocol 2 and 3 had extended processes and overall standard morphology after 12 days in culture (Figure 9E) (Final protocol in Appendix 2). More importantly, cultures prepared from Th-EGFP mice contained EGFP expressing neurons, which indicates that dopamine neurons survive and grow neurites in the culture conditions used (Figure 9F).

As previously reported (Jomphe et al., 2005; Matsushita et al., 2002), the expression of EGFP co-localises sufficiently with Th, and the proportion of Th-immunoreactive neurons increases over time in culture. This property was evaluated with the culture method, by using Th-immunocytochemistry which also permitted the investigation of the coating procedure (Figure 9G). However, the immunocytochemistry experiments showed that none of the tested coating materials provided sufficient support for the monolayer when the coverslip was transferred from the culture dish. The ruptured monolayer prevented statistically valid quantification experiments, yet allowed a qualitative investigation of the

intact regions. Fortunately, this was not an issue with the electrophysiological recordings as they could be conducted with neurons grown directly on the bottom of the dish, which was thus the method of choice for the major part of this project. Eventually, the staining problem was overcome by using the aforementioned inverted staining method for C57BL6 culture, which resulted in higher quality images (Figure 9H). However, owing to time schedule and limited availability of Th-EGFP pups, this was not implemented for other experiments. Regardless, the immunocytochemistry results showed a significant colocalisation of EGFP and TH in Th-EGFP derived cultures, and colocalisation of neuron specific tubulin β3 and TH in C57BL/6J cultures.

With then acute dissociation model, the first goal was to optimise the brain slice preparation to achieve viable neurons (Table 4). Out of the tested parameters, the most efficient method was to use younger P0-P7 animals, and to create the brain slices in ACSF in RT. The highest level of neurons was obtained when the mechanical dissociation was carried out in poly-L-lysine coated culture dishes and when the movement was achieved by vibrating a pulled and subsequently fire-polished micropipette at 30 Hz for longer than 1 min. During this, the tip was moved through the entire slice. In the optimised method, vibrodissociation was powered by a relay connected to a Kenwood pulse generator. While the other tested options resulted in random unidentifiable particles or large aggregates of neurons and connecting tissue, the described protocol yielded viable neurons with reasonably well-preserved synaptic boutons (Figure 9B).

Despite of the promising results, no recordings were obtained from the acutely isolated neurons. This was mostly due to the fact that despite the incubation prior to examination, the cells moved away whenever approached with the electrode micropipette. Thus, further physiological experiments were carried out with cultured VTA neurons.

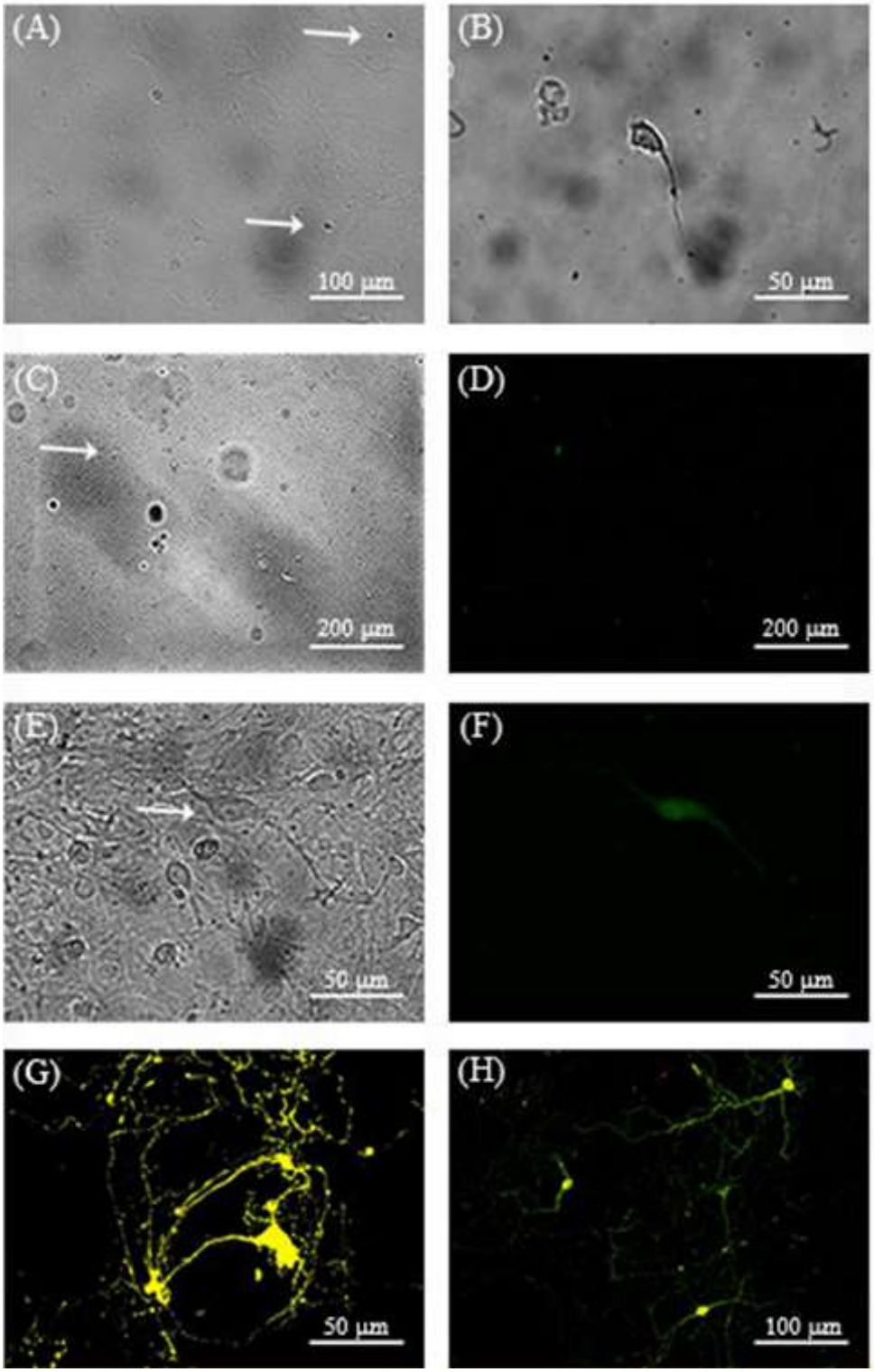


**Figure 9 Primary culture and acute dissociations as models for VTA.** Co-culture was established by creating a purified monolayer of astrocytes (A). The white arrows identify the sparse remaining microglia. Living acutely dissociated neurons in phase contrast C) and during epifluorescence (D) after two hours of dissection and plating onto the monolayer. Living neurons after 12 days in culture in phase contrast (E) and during epifluorescence (F). White arrows identify the dopaminergic neurons. Th-EGFP derived cultures were fixed and immunostained against TH (G). C57BL/6J derived cultures fixed and immunostained against TH (568) and tubulin β3 (488) using the inverted method (H).

**Table 3 Summary of the optimal parameters for mechanical dissociation.**

| Brain slice preparation | | | | |
|---|---|---|---|---|
| Parameter | Age | Cutting solution | Temperature | Coating |
| Optimal | P0-P7 | ACSF | RT | poly-L-lysine |
| **Vibrodissociation** | | | | |
| Piezoelectricity | Pulse genetor | Time/Frequency | Tip shape | Tip position |
| Relay | Kenwood | 1min, 30Hz | Sharp + fire-polished | passing through |

## 5.3. Cultured VTA neurons show response to glutamate

Ventral mesencephalic neurons from C57BL/6J or Th-EGFP mice were cultured together with cortical astrocytes for 10-15 days prior to recordings. The cultures then received glutamate with increasing concentrations, and the responses were recorded (Figure 10A and 10C). The results however, did not represent the expected saturating concentration-dependent curves for either of the mouse strains, albeit only one response deviated in both experiments. Amplitudes of the dose responses were higher for Th-EGFP derived culture. It is noteworthy that only one recording was achieved from each strain. Owing largely to mechanical problems of the equipment, the patches were unstable and thus failed recordings. After attempts to alter the culture conditions, or to modify the perfusion system to stabilise the solution level did not suffice, it was suspected that the problem was due to unstablility of the micropipette holder. But eventually, the problem was resolved by changing the inner rubber ball that leaked the pressurized air needed to dampen vibrations of the recording platform. After this, the patches remained sufficiently stable to measure the full dose responses but for the sake of schedule, only two recordings were acquired. Nevertheless, the acquired traces resembled excitatory postsynaptic currents mediated by AMPA receptors (Figure 10B).

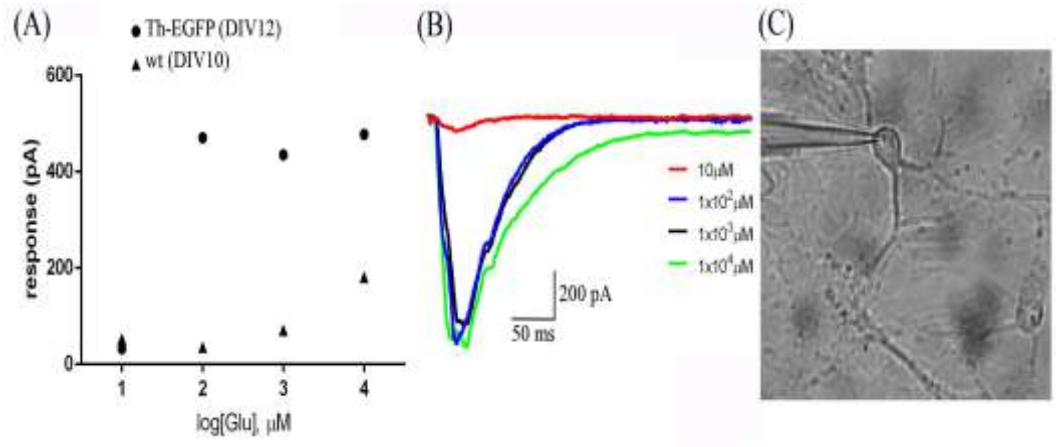


**Figure 10 VTA DA neurons show non-linear dose response mediated by AMPA receptors.** Glutamate was applied to cultured wt and Th-EGFP neurons in increasing concentrations: 10µM, 100 µM, 1mM and 10mM. The traces were averaged for each agonist concentration (A) and the averaged responses were plotted against time (B) from patched DA neurons in whole cell voltage clamp at -70mV(C).

# 6. Discussion

On the basis of the presented tyrosine hydroxylase expression map, it can be concluded that the distribution of DA neuron populations is equal in adult and young animals and hence there might be less requirement to use older animals for the experiments. However, since studies have shown that the regulation of brain circuits is often development-dependent (Bellone and Lüscher, 2012), implementing adult controls should be taken into consideration. In addition, the Th-EGFP line shows an expected A16-A8 phenotype, and subpopulations of DA neurons can also be identified. The relatively high standard error of the means present in some of the average cell numbers could be due to partially damaged brain slices or it could be a result of an uneven slicing of the brain samples. However, the differences were not significant between the two age groups. Overall, this strain provides an excellent model for identifying and studying the characteristics of DA neurons.

The explanation for the higher yield of astrocytes obtained with papain digestion, could be that the washing steps were not sufficient to abolish all trypsin activity or that the incubation period was too long, thus resulting in unnecessary degradation of the tissue. Based on the previous findings (Banker & Goslin, 1998), astrocytes attach readily on plastic surface and have fewer demands for the culturing conditions. Thus, it was not surprising to find that the choice of coating or media supplements had minimal difference on the growth of the cells especially when the culture was plated directly on to the bottom of the dish. Despite of this, the monolayer grown on glass coverslips could not resist the transfer-related mechanical stress. According to the literature, this issue could have been overcome by pre-coating the coverslips with collagen (Fasano et al., 2008; Jomphe et al., 2005). Unfortunately, this reagent was not available for this project. The inverted staining method solved this issue, but as it prevents the appropriate storing of the samples and involves a risk of damaging the microscope unless the edges of the dishes are removed, it should be replaced with suitably coated coverslips in the future.

GDNF has been proposed to be important for the survival and differentiation of DA neurons (Lin et al., 1993). In accordance with these findings, protocols which involved the reagent provided the highest level of viable cells. It should be noted, however, that exogenous GDNF has been reported to block certain behavioural and biochemical responses to cocaine and morphine (Messer et al., 2000). Kynurenic acid, on the other hand, is considered to prevent excitotoxicity and might thus be a beneficial addition to protocol 2. However, it acts by blocking glutamate receptors (Fasano et al., 2008). While it is improbable that the duration of either effects would last as long as 12 days, a control culture without these reagent should always be prepared for physiological studies to assure that the results are not significantly altered.

The other reason why a higher level of viable neurons was obtained with protocol 2 and 3 might be due the preconditioning step that these protocols included. In this step, astrocyte medium was replaced with Neurobasal-A prior to neuronal plating. This has been shown to be beneficial for other types of neurons (Kaech & Banker, 2006). Even though this theory was also exploited in protocol 1 by supplementing the neuronal medium with the collected and conditioned Basal Medium Eagle Minimum or Essential Medium, it was perhaps not sufficient to create an appropriate surrounding for the sensitive DA neurons. On the other hand, feeding the co-cultures without any astrocyte-adjusted medium could be problematic in the long-term growth. However, this was not an issue within the time frame of this project. In the future, if the purpose is to carry out long-term investigations it could be beneficial to decrease the media change interval for the co-culture because the culture dishes had larger diameters in the protocol published by Fasano et al., (2008).

The differences of the cultures obtained by the alternative purification protocols might be misleading because the percentage of DA neurons has not been statistically quantified. Overall, the comparison is challenging due to the fact that one of the most critical factor influencing the neuron survival is the rate of the dissection and tissue handling (Fasano et al., 2008). Thus, the results may alter significantly depending on the experience level of the individual performing the procedures; hence the viability of the neurons was overall higher towards the end of

this project. In the future, the culture preparation should always be prepared in teams of two. Regardless, the immunocytochemistry experiments validate the set up protocol as a suitable *in vitro* model for VTA DA neurons. Yet, more optimisation followed by appropriate staining methods are required to assure that the DA percentage is sufficiently high, and that the level is consistent in every preparation. The accuracy of the dissection procedure is also dependent of the experience level. Thus, molecular markers and pharmacological properties of the cultured neurons should be investigated to assure that the A9 region is sufficiently excluded from the preparation.

At this stage, primary culture was advantageous over acute dissociation. The difficulties of patching attributable to the inadequate neuron attachment were possibly caused by transferring the culture dish from the vibrodissociation set-up to the electrophysiology equipment. Previously, all of these procedures have been reported to take place in the same location (Jun et al., 2011). Unfortunately this was not possible with the available equipment. On the other hand, cell contact could have been strengthened by adding a collagen pre-coating (Jomphe et al., 2005) or by increasing the incubation time. However, as the perfusion system was not used at this step, longer incubation times could have decreased neuronal survival as media replenishment was not possible.

Despite the recommendation for primary culture, the plausible impact of collagen was somewhat doubtful for this method since mechanical isolation has been achieved even without the use of any coating material. On the other hand, the difficulty in obtaining patches might be due to the choice of species as several studies have been conducted with rats (Vorobjev, 1991; Ye et al., 2004). Since the yield of healthy neurons is often relatively low in acute isolations (Jun et al., 2011), achieving a sufficient rate of data accumulation could have required more experience in brain slice preparation. Regardless, considering that the optimal protocol involved P0-P7 animals and the inaccuracy of the available micromanipulator, the exact dissociation of mere VTA was less ambiguous by scalpel and free hand.

Whole-cell recordings obtained from the cultured neurons further validate the

set-up protocol as a sufficient method to achieve viable DA neurons. The acquired traces were deduced to originate from AMPA receptors based on their kinetics. The rapid rising phase and deactivation are stereotypic for these low-affinity binding site bearing receptors, whereas NMDA receptors show longer activation time course (Dingledine et al., 1999; Möykkynen & Korpi, 2012). Furthermore, the holding potential was retained at -70 mV throughout the experiment, which maintains the NMDA block (Nowak et al., 1984). In the future, the responses could be confirmed to originate from AMPAR by applying the CNQX-antagonist to the cells and then investigating whether the response is successfully blocked. The unexpectedly high second response in Th-EGFP neuron could be caused by minor instability present in the micropipette, or noise from the environment. On the contrary, blockade in the fast applicator tube could have elicited the unexpectedly high second response in Th-EGFP neuron recording. This hypothesis is supported by the fact that out of the 10 traces recorded with this concentration, the last 3 already activated minor responses. Nonetheless, if a higher amount of recordings had been obtained, the averaged results could have attenuated these outliers.

The dose response curves looked different between the two different mouse strains. This could be due to the ages of the cultures, or the sizes of the cells or that they were different subclasses of DA neurons. Alternatively, Th-EGFP neuron could in fact be a glutamatergic neuron co-expressing Th or most presumably, as it is not possible to visually distinguish live wild-type neurons and the $I_h$ current was not recorded, the recording from wt culture could in fact be from a different type of cell because VTA contains 30% GABAergic and 2-3% glutamatergic neurons (Ungless and Grace 2012; Fasano et al., 2008).

# 7. Conclusions

The Th-expression map showed that 3-week-old animals show mature DA neuron distribution. The comparison of A10 *in vitro* models showed that at this stage, the reduced preparation time and plausible unaltered electrical properties presented by the mechanical dissociation did not compensate the required additional efforts to optimise the method further. Thus, a higher yield of viable VTA DA neurons was obtained with the primary culture particularly when astrocytes had been obtained with papain and enriched through cold wash and neurons had been purified without centrifugation and supplemented with GDNF.

In essence, primary astrocyte neuron co-culture combined with the Th-EGFP mouse strain is an excellent *in vitro* model for studying VTA DA neuron physiology, and even if no protocol is without its drawbacks, the initial glutamate responses imply that such methodology was successfully set up during the course of this thesis. In the future, the technique could be further developed for other DA populations or multiple brain region co-cultures.

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# Appendix 1. Photographs of the electrophysiology and mechanical isolation equipment

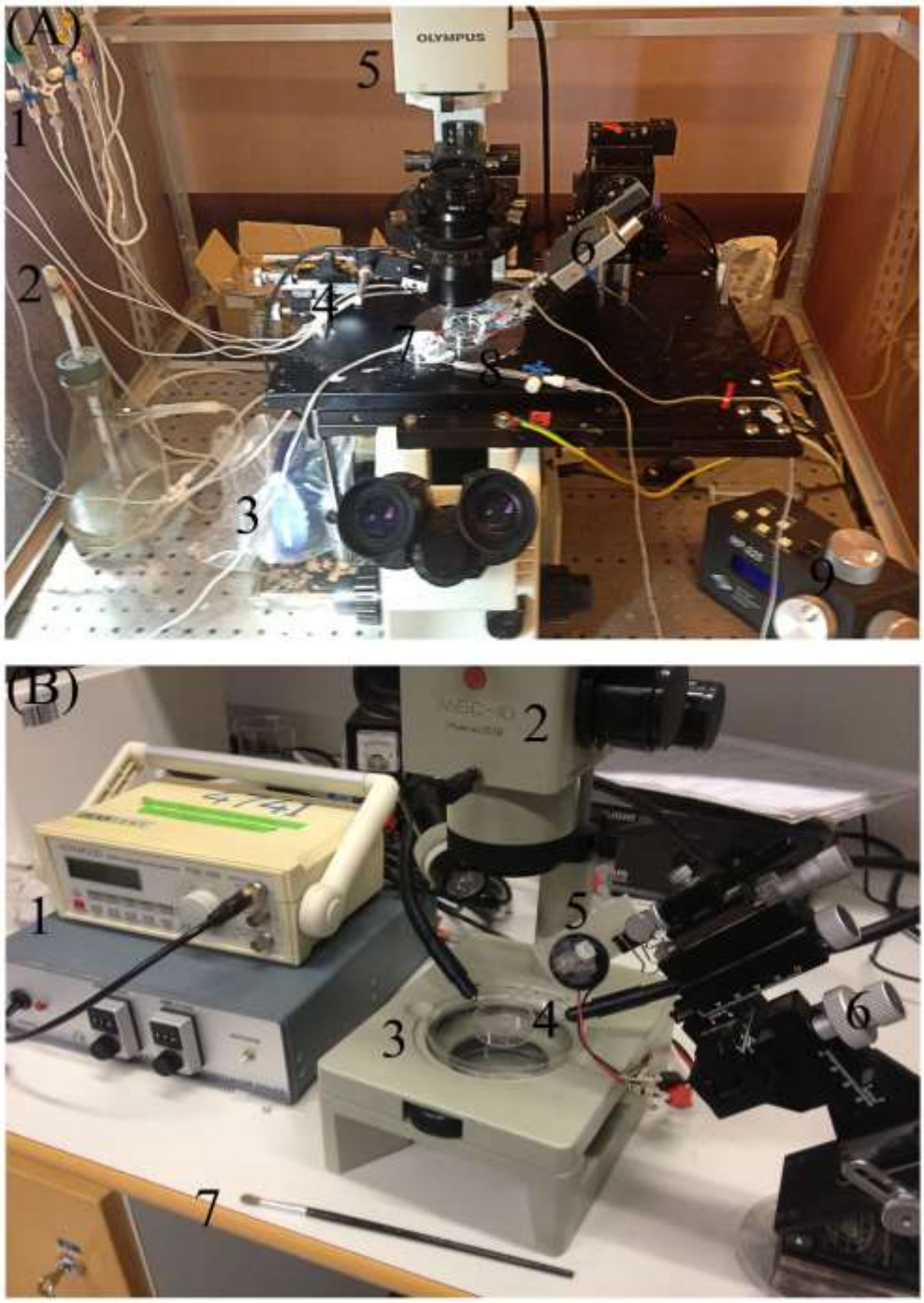


**Figure 11 Photographs of the electrophysiology and mechanical isolation equipment.** In patch clamp (A) culture dish was placed onto camera (3) equipped microscope (5) and extracellular solution was constantly applied to the dish through the general perfusion system (7) and removed by the pump (2). Agonist of increasing concentrations was directly applied to the cell through fast-step applicator system (4). A micropipette was attached to the electrode holder (6) connected to a micromanipulator (9) and a mouthpiece (8) was used to patch the neurons. In vibrodissociation (B) slices were transferred to a culture dish (3) with a brush (7) placed on a microscope (2) stage. Manipulator (6) connected micropipette (4) was positioned and vibrated by a pulse generator (1) connected piezoelectric component (5).

# Appendix 2. Protocol for postnatal ventral midbrain dopamine neuron culture

**Preparing the astrocyte culture (1 x P0-P5 pup of any strain or gender)**

- Prepare 5 ml papain solution and activate it, warm 50 ml of astrocyte medium
- Place 2 ml of dissociation solution in a 100 x 15-mm culture dish
- Sterilise all the dissection instruments and aluminium foil square using 70 % ethanol
- Decapitate pup, allow the head fall onto aluminium foil square and move to fume hood. Place the brain on the culture dish
- Under the binocular microscope, place the brain with dorsal side facing upward, remove the olfactory lobes and the diencephalon. Peel off meninges (**Be thorough,** meninges contain fibroblasts that can overgrow astroglial cultures). Isolate the cortex and cut it into $1mm^3$ chunks using forceps
- Place tissue pieces in papain and incubate for 20-30 min at 37 °C
- Wash the tissue blocks 3 x 2.0 ml of astrocyte media, replace with 1 ml of media and begin triturations: Triturate 20 times using 5 ml pipette and 40 times with 1000 µl pipette
- Pellet the glial cells at 1000 x g for 5 min, resuspend pellet in 15 ml Glial medium. Plate the cells into a 75-$cm^2$ culture flask
  (The yield should be approximately $7.5x10^6$ cells)
- After 24 h in culture, wash the cells twice with cold medium and vigorous shaking to dislodge the neurons and microglia
- Feed the culture every 2-3 d, replace half of the medium and slap the flask to dislodge loosely attached cells.

**Coating of 22-mm coverslips**

- Incubate coverslips for 24 hr in 12 N HCl
- Rinse 3 x $ddH_2O$ and leave in $ddH_2O$ for 1 hr
- Drop the coverslips into a beaker containing 100 ml 95% EtOH, leave for 1min
- Shake off the excess EtOH, place delicately on sterilized Whatman paper and store in an airtight box
  (Surface must be perfectly transparent, storage up to several weeks)
- Cover the surface of each coverslip with of collagen solution (100 µl) per coverslip and let dry completely
- Add 100 µl of poly-L-lysine solution, incubate for 1 h
- Rinse by sequentially plunging the coverslips into 3 beakers of $ddH_2O$. Place the coverslips on sterilized Whatman paper and place 100 µl $ddH_2O$. Incubate for 1 h and repeat. Incubate 1 h and aspirate off excess $ddH_2O$. Let dry completely. **Make sure to rinse thoroughly**, excess poly-L-lysine can be toxic to the
- Grow until confluence (7-10 d)

**Preparing the glial feeder layer (after 7-10 d in culture)**

- Place one coated coverslip to each 35 x 10mm well and add 2 ml of astrocyte media. Place into 37 °C 1-2 h before plating the cells
- Aliquot 21 ml of D-PBS and incubate at 37 °C
- Wash the culture 2 x 6ml D-PBS
- Add 2 ml of trypsin to the culture flask and incubate at 37°C until the first cells begin to lift off (usually less than 2 min). Add 5ml astrocyte medium to stop the trypsinisation, pipet repetitively and transfer the cell suspension to 15ml tube
- Centrifuge (1000g, RT, 5 min)
- Resuspend in 10 ml, count the cells using hemocytometer and plate at a density of 100000 cells/ml
- Once a uniform monolayer of astrocytes is formed, add 12 µl of FUDR solution per dish.

**Preparing the neuronal culture (P0-P2 mouse pups) (work in pairs: one harvesting brains the other isolating the VTAs)**

- 1 day before the dissection replace the astrocyte medium with 2ml Neurobasal A/B27 supplemented with
  (Preconditioning is important for optimal neuronal growth)
- Prepare 5 ml papain solution and activate it, warm 50 ml of neuronal media
- Prepare one 15 ml tube of dissociation solution per brain plus one extra for midbrains, keep on ice.
- Sterilise all the dissection instruments using 70 % ethanol
- Decapitate pups, allow the head fall onto aluminium foil square and move to hood
- Collect the brains on 15 ml tubes
- Hold the brain ventral side up, remove the cerebellum, move 3mm in the anterior direction and do another cut at the mammillary bodies
- Move the piece so that the anterior side faces up
- Remove the segments dorsal, ventral and lateral to VTA and remove residual meninges
  (Perform the dissection in less than 1.5 min to minimize neuronal damage)
- Cut the remaining piece in two, and then in smaller segments, collect the segments in, transfer the pieces to papain and incubate for 20-30 min at 37 °C
- Using a plastic Pasteur pipette, transfer the brain segments into a sterile 15 ml tube (try to avoid excess papain solution) and wash them 3 x 2 ml with warmed neuronal medium: allow segments to settle after each media addition and then carefully **remove** as much solution as possible without disturbing the segments. Change pipettes to avoid contamination!
- Begin triturations in 2ml of neuronal media. Triturate 25 times with 5 ml pipette (avoid letting in air bubbles) and let the tube sit for 3 min until undissociated segments settle. Use a transfer pipette to remove and **keep** as much sol'n as possible without disturbing segments at the bottom. Keep the supernatant in a new 15m tube
- Repeat the previous step using a 1000µl pipette and 200µl pipette until completely dissociated
- Pour the lysate through a 40-µm nylon strainer (BD Biosciences)

- Count the cells. Multiply the number of cells by 10. This is the number of cells/µl. Divide by 240 000 (or desired density) by this number. This is the number of µl to add per dish
- Add 100 µl of GDNF after plating and 10 µl of FUDR after 24 h in culture per dish. Disturb the dishes as little as possible for the next 7 d
- Change one-third of the medium one week after plating and add 10 µl of kynurenic acid per dish. Change one-third of the medium (1:2 neuronal and astrocyte medium) every week thereafter
- Maintain for 10-18 days in culture prior to electrophysiological experiments

**Solutions (reagents can be obtained from Sigma, unless otherwise indicated)**
(Obtained and modified from Fasano et al., 2008, GDNF recipe obtained and modified from Frank et al., 2008)

**Collagen solution (for 50 22-mm coverslips)**
- 7.25 ml dd$H_2O$
- 250 µl PureCol (3.0mg/ml INAMED Biomaterials) or equivalent reagent
- Use freshly made

**Borate buffer for poly-L-lysine (store up to 3 weeks at 4 ºC)**
- 50 ml of dd$H_2O$
- 155 mg $H_3BO_3$
- 238 mg $Na_2B_4O_7$ decahydrate
- Adjust pH to 8.5 with 1 N HCl filter-sterilize

**Dissociation solution (store up to 1 month at 4 ºC)**
- 6.39 g $Na_2SO_4$
- 2.62 g $K_2SO_4$
- 590 mg $MgCl_2$ hexahydrate
- 18.4 mg $CaCl_2$ dihydrate
- 1.19 g HEPES
- 1.80 g D-glucose
- Add ddH2O to 500 ml
- Adjust to pH 7.4 with 1 N NaOH and filter-sterilize

**GDNF solution (prepare 76.9 µl aliquots and store up to 1 year at −20ºC)**
- 5 µg GDNF (Life Technologies)
- 2.4 ml dd$H_2O$
- Dilute to working solution with 723.1 µl neuronal media (for 8 culture dishes)

**FUDR solution (prepare 1 ml aliquots and store up to 1 year at −20ºC)**
- 203 ml MEM
- 100 mg 5-fluoro-2-deoxyuridine
- 198 mg uridine
- Filter-sterilize

**Kynurenic acid (prepare 1 ml aliquots and store up to 1 year at −20ºC)**
- 10 ml dd$H_2O$
- 236.5 mg kynurenic acid

- Add 5 N NaOH until powder is dissolved and filter-sterilize

**Astrocyte medium (store up to 1 week at 37ºC)**
- 85.7 ml Minimum Essential Medium (with Earle's salts, without L-glutamine and phenol red; Invitrogen)
- 1 ml D-glucose MEM (18.02 g D-glucose into 50 ml of MEM, store up to 3 months at 37 ºC)
- 1 ml penicillin/streptomycin (100× stock solution from Invitrogen)
- 1 ml GlutaMAX (Invitrogen)
- 1 ml of 100 mM sodium pyruvate
- 100 µl MITO+ (Invitrogen) (1 vial of MITO+ into 5 ml of $ddH_20$, store up to 1 year at -20 ºC)
- 200 µl phenol red solution, 0.5% in D-PBS
-Filter sterilize
-10 ml FBS

**Neuronal medium (store up to 1 week at 37ºC)**
- 86 ml Neurobasal-A medium (without L-glutamine)
- 1 ml penicillin/streptomycin (100× stock solution from Invitrogen)
- 1 ml GlutaMAX
- 2 ml B27 supplement
- Filter sterilize
- 10 ml FBS

**Papain solution (store at 37ºC for a maximum of 30 min)**
- 5 ml dissociation solution
- 2.25 mg L-cysteine-HCl monohydrate
- Adjust to pH 7.4 with 1 N NaOH
- 10 mg papain
- Activate at 37ºC for 15 min, filter-sterilize and use freshly made

**Poly-L-lysine (prepare 200 µl aliquots and store up to 2 years at −20ºC)**
- 5 ml borate buffer for poly-L-lysine (see recipe)
- 5 mg poly-L-lysine hydrobromide (Sigma)
- Dilute to working solution with 1.8 ml of borate buffer (for 15 coverslips).